APPLICANT(S):	WALDMANN, Herman , et. al.		
SERIAL NO.	09/849,499	EXAMINER:	TON, Thaian N.
FILED:	May 4, 2001	GROUP ART UNIT:	1632
FOR:	METHOD FOR PRODUCING DENDRITIC CELLS		

DECLARATION UNDER RULE 37 C.F.R. 1.132

I, Paul Fairchild, a citizen of the United Kingdom, residing at 30 Carlton Road, Oxford, OX2 7SB, hereby declare:

1. I am a University Research Lecturer at the Sir William Dunn School of Pathology, University of Oxford, United Kingdom. My field of expertise is in immunology and stem cell biology. My Curriculum Vitae and list of publications are attached herewith as Appendix 1.

2. I have reviewed the subject Application and the Office Action dated April 9, 2003, issued by the United States Patent and Trademark Office in connection with the subject Application. Claim 64 claims:

> "[a] process for producing a long-term culture of immature dendritic cells, which process comprises: culturing the embryonic stem (ES) cells in the presence of a composition comprising IL-3, which brings about differentiation of the ES cells into immature dendritic cells, whose protracted longevity and capacity for self renewal produce a long-term culture of immature dendritic cells; and recovering the said immature dendritic cells from the culture, which immature dendritic cells are capable of maturation to an immunostimulatory phenotype."

Furthermore, new claim 110 claims:

> "[T]he method of claim 64, which said composition further comprises GM-CSF."

3. In the Office Action, the Examiner rejected the claims under 35 USC 112, first paragraph. Specifically, the Examiner asserted that the specification allegedly fails to provide an enabling disclosure for utilizing any other ES cell line.

4. The Examiner is incorrect in the basis for the rejection. The application discloses and teaches one skilled in the art how to make dendritic cells from mouse or human ES cells. The examples provided in the application show that ES cells derived from at least three mouse strains were effective in differentiating into dendritic cells:

> "[i]n support of the latter possibility, initial studies on the CBA/Ca cell line ESF116 were repeated using a second CBA/Ca line generated in-house (ESF99) and one from 129/Sv mice, which is widely used for gene knockout technology and which is commercially available. Interestingly, while ESF99 supported the development of esDC, albeit to a lesser extent than ESF116, D3 failed entirely to do so under the same culture conditions. ES cells generated from other strains can easily be tested for their ability to support development of DC by using the protocols described herein. An additional example of a mouse strain from which ES cells have been shown to support development of DC is C57BL/6 (ESF75)". [Page 8 lines 19-31]

5. Furthermore, we have extended our work to several other mouse ES cell lines, each of which behaves identically with respect to the production of dendritic cells. Furthermore, Cheng *et al.* [*Blood* (2003) **102**:3980-3988] have reported the successful use of a commercially available mouse ES cell line derived from 129 mice (R1) for the generation of dendritic cells according to our published protocols.

6. Since, as demonstrated in the application, at least three mouse ES cell lines (ESF99, ESF116 and ESF75) were effective in producing a long-term culture of immature dendritic cells, by culturing the ES cells in the presence of a composition comprising IL-3, one skilled in the art would be enabled to practice the invention without undue experimentation, namely to generate dendritic cells from any mouse ES cell line.

7. In addition, it is known to those skilled in the art that there is significant conservation of the developmental processes between mouse and human, particularly with respect to hematopoiesis. Accordingly, based on the disclosed methods, materials and experiments, one skilled in the art can expect to obtain dendritic cells from human ES cell lines.

8. It is noted that it has been difficult to obtain human ES cells in both Europe and the United States to conduct such work due to ethical and regulatory restrictions.

9. The derivation of dendritic cells from human ES cells was demonstrated by Zhan *et al.* (2004) [*Lancet* **364**:163-171] who generated a broad range of hematopoietic cell types from human ES cells, including dendritic cells. Zhan *et al.* state that "to generate a broad range of haematopoietic cells, including dendritic cells and other antigen-presenting cells, we adapted a protocol developed previously for mouse ES cells" [p164, column 2, paragraph 3] and cite Fairchild *et al.*, (2000) [*Curr. Biol.* **10**:1515-1518] in this respect, which method corresponds to the method of the subject application. Zhan *et al.* apply the protocol for the production of dendritic cells, using IL-3 as claimed in the subject application. In order to broaden the range of leukocytes obtained, so as to make their results applicable to their stated objective, Zhan *et al.* added a number of other growth factors, namely (i) stem cell factor, (ii) Flt3 ligand and (iii) thrombopoietin, each of which they acknowledge to be "widely used to maintain human postnatal haematopoietic stem cells and to expand committed progenitor cells" [p164; column 2, paragraph 3], in

accordance with the literature [Luens *et al.* (1998) *Blood* **91**:1206-1215; Koizumi *et al.* (1998) *Exp. Hematol.* **26**:1140-1147]. The addition of IL-4 to the culture medium was purely to "enhance possible maturation of lymphoid cells and dendritic cells" [p165, column 1, lines 1-2].

10. Thus, Zhan *et al.* obtain dendritic cells from human ES cells based on the method disclosed in the subject application.

11. Therefore, contrary to the Examiner's assertion, the subject application is enabled for obtaining dendritic cells from any mouse or human ES cells.

The undersigned further declares that all statements made herein of his own knowledge are true, and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made, are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date: October 21, 2004

Paul Fairchild

Curriculum Vitae

Paul Jonathan Fairchild

Personal Details

Date of Birth:	18 April 1965
Nationality:	British
Marital Status:	Married
Home Address:	30 Carlton Road, Oxford, OX2 7SB
	E.mail: Paul.Fairchild@path.ox.ac.uk
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Employment History

2001 – Present:	University of Oxford, Sir William Dunn School of Pathology
	University Research Lecturer
1995 - 2001:	University of Oxford, Sir William Dunn School of Pathology
	Senior Scientist (RSII)
1999 - 2000:	University of Oxford, Oriel College
	College Lecturer in Medicine
1991 - 1995:	University of Cambridge, Department of Pathology
	Post-Doctoral Research Associate (RA1A)

Education

1987 - 1991:	University of Oxford, Wolfson College,
	Linton Road, Oxford, OX2 6UD
1983 - 1987:	University of Leicester, University Road,
	Leicester, LE1 7RH
1981 - 1983:	Haywards Heath Sixth Form College,
	Harlands Road, Haywards Heath, West Sussex
1976 - 1981:	Chailey Comprehensive School, Mill Lane,
	South Chailey, West Sussex

Academic Qualifications

DPhil (Oxon)	*Title of Thesis*		
	'The Role of Dendritic Cells in Selection of the T Cell Repertoire'		
BSc (Hons)	Biological Sciences	-	Class I Honours
	Subsidiary		
	Mathematical Statistics	-	Class I with Distinction
	Award		
	M. Wallace Henry Award for top graduate of Biological Sciences		
'A' Levels	Biology	-	Grade A
	Spanish	-	Grade A
	Mathematics	-	Grade B
GCE 'O' Levels	Grade A obtained in 10 of 12 subjects		

Professional Activities

Appointments

- Advisor to the Netherlands Government Central Committee for Research Involving Human Subjects: 2000 - Present
- Elected member of the Local Management Committee of the Whitefield Institute: 2000 - 2002
- Invited member of Faculty and International Advisory Committee for the 6th International Conference on *New Trends in Immunosuppression*
- Appointed assessor for the Part I Molecular and Cellular Biochemistry examination: 2003
- Appointed examiner for the theses of six PhD candidates

Recognition

- Awarded MA status by the University of Oxford and admitted to congregation: 1999
- Entry in *Who's Who in Medicine and Healthcare*: 2003
- Departmental Merit Award for achievements in research and teaching: 2003

Invitations from Scientific Organisations

- Regular reviewer of grant applications submitted to the following charities:
    - The Wellcome Trust
    - Institut National de la Santé et de la Recherche Médicale (INSERM)
    - The Swiss National Science Foundation

- Guy's & St Thomas' Hospital Trust
- Action Research
- The Biotechnology & Biological Sciences Research Council (BBSRC)
- Child Health Research Appeal Trust (CHRAT)

- Regular reviewer of manuscripts submitted to the following journals:
  - *Nature Medicine*
  - *Immunology*
  - *Journal of Leukocyte Biology*
  - *Journal of Anatomy*
  - *Journal of Autoimmunity*
  - *Blood*
  - *Transplantation*
  - *The Lancet*
  - *Trends in Immunology*
  - *Journal of Cell Science*
  - *International Journal of Cancer*
  - *European Journal of Immunology*
  - *Scandinavian Journal of Immunology*
  - *International Immunopharmacology*
  - *Therapeutic Immunology*
  - *Clinical and Experimental Immunology*
  - *Medical Science Monitor (Reviewers Panel)*
  - *American Journal of Transplantation*

Teaching and Lecturing Experience

Lecturing

- Lecturer for the first year degree course in Physiology: 1998 - Present
- Lecturer for the second year degree course in Biochemistry: 2001 - Present
- Lecturer for the third year (FHS) Immunology Option: 1998, 2001 – Present
- Module leader for the MSc in Immunology: 2004

Tutoring:

- College Lecturer in Medicine, Oriel College, covering sabbatical leave: 1999 - 2000
  - Responsible for organisation, delivery and administration of medical teaching throughout the College
  - Tutor of Cell Biology (1st BM Part I), Pathology (1st BM Part II) and Immunology (FHS Option)
  - Pastoral care of pre-clinical students
  - Setting and marking of termly collection papers
- Tutor for the FHS Immunology Option, Sir William Dunn School of Pathology, University of Oxford: 1996 - Present
- Tutor of Immunology for the Part II Medical and Veterinary Sciences Tripos, Department of Pathology, University of Cambridge: 1992 - 1995

- Tutor of Pathology, Downing College, University of Cambridge:

1991 - 1995

Demonstration of Practical Classes

- Demonstrator of practicals for the 1st BM Part II: University of Oxford 1996 - 2000
- Demonstrator of practicals for the 2nd year degree course in medicine: 1992 - 1995
- Design, planning and implementation of practical classes for Part II of the Medical and Veterinary Sciences Tripos: University of Cambridge 1994 - 1995

Supervision of Research Projects:

- Supervision of 3 PhD students: 1997 - Present
- Supervision of 8 undergraduate research projects (6 awarded class I): 1993 – Present

Invited Lectures

Institutions

- Nuffield Department of Surgery, University of Oxford
- Department of Surgery, University of Cambridge
- Department of Immunology, The Babraham Institute
- Department of Animal Sciences, University of Reading
- The Basel Institute for Immunology
- Department of Pathology, University of Cambridge
- Sir William Dunn School of Pathology, University of Oxford
- Centre for Genome Research, University of Edinburgh
- Imperial College School of Medicine, University of London
- Northwick Park Hospital, Imperial College, University of London
- Synaptica Ltd, Harwell International Business Centre, Oxon
- Department of Human Anatomy & Genetics, University of Oxford
- London School of Hygiene and Tropical Medicine
- Christian Medical Fellowship
- Max-Plank Institute for Infection Biology, Berlin

Personal Invitations to Present at Conferences:

- Royal Society of Medicine: London 2005
  'Recent Developments in Stem Cell Research'
- Euroscicon Conference: London 2004

'Dendritic cells: Identification, Isolation, Expansion and More'

- The Medical Research Council Stem Cell Initiative: Warwick 2004
- 6th International Conference on Immunosuppression: Salzburg 2004
  Chairperson for session on 'Advances in Clinical Immunosuppression'
- XIX International Congress of Transplantation: Miami 2002
  'State of the Art Symposium on Dendritic Cells'
- Oxford Science Day on 'Stem Cell Biology': Oxford 2002
- BSI Congress: Harrogate 2001
  'From Cloning to Stem Cells: New Directions in Transplantation'
- The Gulbenkian Science Institute Autumn Meeting: Lisbon 2000
  'Cellular Differentiation'
- Trinity College, Oxford: 2000
  'From the Laboratory to the Clinic'
- Mérieux Foundation International Symposium: Annecy 2000
  'Pluripotent Stem Cells: Therapeutic Perspectives and Ethical Issues'
- Oxford Immunology Group One Day Symposium: 1999
  Chairperson for session on Antigen Presenting Cells
- European Research Conference: Blankenberg 1996
  'Therapeutic Immunomodulation'
- Oxford Series in Biomedical Sciences: 1996
  'Autoimmunity: From Understanding to Therapeutics'
- British Society for Allergy and Clinical Immunology: 1992
  'T cells in Disease'
- The British Council: 1991
  'Immunotherapy with Monoclonal Antibodies'

Intellectual Property and External Funding

- International Patent Application (PCT/GB99/03653) filed in November 1998: 'Method for Producing Dendritic Cells from Embryonic Stem Cells'
- Awarded MRC project grant: 'Reprogramming the Immune System to Accept Histoincompatible Stem Cell-Derived Tissues': 2004
- Co-applicant for successful grant application to ROTRF: 2002
- Contributions to the success of an MRC Programme Grant: 1999
- Awarded Royal Society Fellowship with research support for 5 years: 1995

Continuing Professional Development

Workshops Attended

• Essentials of Small Businesses: Venturefest	Jun 2004
• Supervision of Graduate Students: University of Oxford	Mar 2004
• Quality Assurance in the Laboratory: University of Oxford	Jul 2003
• Digital Imaging: University of Oxford	Feb 2003
• Better Teaching & Learning Through C&IT: LTSN Bioscience	Jan 2003
• Managing Research Projects: University of Oxford	Mar 2001
• Tutorial Teaching: University of Oxford	Jan 2000
• Applying for Research Grants: University of Cambridge	Mar 1993

Professional Qualifications and Affiliation:

- Diploma in Therapeutic Counselling (Humanistic)

1996-2000

- Membership of the BACP 2000 – Present

Miscellaneous

- Clean driving licence
- Former fluency in Spanish
- Computer literacy

Publications

Media Coverage of Scientific Research

- Cell success gives hope. *The Times Higher Education Supplement* 12 January 2001 p21 .
- Lords back broader stem cell research. *The Times Higher Education Supplement* 26 January 2001 p56
- Boost for the immune system. *Blueprint* 22 February 2001 p9
- Technique 'could boost immunity'. *BBC News Online*. 9 January 2001
- Stem cell scientists given £40m boost. *The Times* 10 December 2002 p12
- New investment in stem cell therapies. *Blueprint* 3 June 2004 p1
- Framework bolsters stem cell progress. *Cur Biol* **14**:R592-593
- Großbritannien als Paradies der Stammzellforschung. *Nachrichten aus der Chemie* In Press
- Stem cell biology in Oxford. *Oxford Today* In Press

Invited Editorship

- Fairchild, P.J. (Ed.) *Immunological Tolerance; Methods and Protocols*. Humana Press, Totowa USA. In Preparation

Published Articles

- Fairchild, P.J. and J.M. Austyn (1990) Thymic dendritic cells: phenotype and function. *Int Rev Immunol* **6**:187-196
- Smith, R.M. and P.J. Fairchild (1992) Monoclonal antibodies: Harnessing the potential. *Sci Progress* **76**:323-343
- Wraith, D.C., B. Bruun and P.J. Fairchild (1992) Cross-reactive antigen recognition by an encephalitogenic T cell receptor. Implications for T cell biology and autoimmunity. *J Immunol* **149**:3765-3770 [57 citations]
- Fairchild, P.J. and D.C. Wraith (1992) Peptide-MHC interaction in autoimmunity. *Cur Opin Immunol* **4**:748-753
- Wraith, D.C., P.J. Fairchild and B. Metzler (1993) MHC binding peptides as therapeutic agents. *Clin Exp Rheumatol* **11** (Suppl 8):S45-S46

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- Fairchild, P.J. and H.C. Macgregor (1994) Lampbrush chromosomes: asymmetric loops. *Cur Biol* **4**:919
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- Fairchild, P.J. and J.M. Austyn (1995) Developmental changes predispose the fetal thymus to positive selection of $CD4^{+}CD8^{-}$ T cells. *Immunol* **85**:292-298
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- Liu, G.Y., P.J. Fairchild, R.M. Smith, J.R. Prowle, D. Kioussis and D.C. Wraith (1995) Low avidity recognition of self-antigen by T cells permits escape from central tolerance. *Immunity* **3**:407-415 [Joint first authorship] [167 citations]
- Fairchild, P.J. and D.C. Wraith (1996) Lowering the tone: mechanisms of immunodominance among epitopes with low affinity for MHC. *Immunol Today* **17**(2):80-85 [45 citations]
- Brocke, S, K. Gijbels, M. Allegretta, I. Ferber, C. Piercy, T. Blankenstein, R. Martin, U. Utz, N. Karin, D. Mitchell, T. Veromaa, A. Waisman, A. Gaur, P. Conlon, N. Ling, P.J. Fairchild, D.C. Wraith, A. O'Garra, C.G. Fathman and L. Steinman (1996) Treatment of experimental encephalomyelitis with a peptide analogue of myelin basic protein. *Nature* **379**:343-346 [262 citations]
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- Fairchild, P.J. and D.C. Wraith (1997) Do epitopes derived from autoantigens display low affinity for MHC class I? *Immunol Today* **18** (2):98
- Fairchild, P.J. (1997) Altered peptide ligands: prospects for immune intervention in autoimmune disease. *Eur J Immunogenet* **24**(2):155-167
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- Fairchild, P.J. (1998) Reversal of immunodominance among autoantigenic T-cell epitopes. *Autoimmunity* **30**(4):209-221
- Fairchild, P.J. and H. Waldmann (2000) Extrathymic signals regulate the onset of T cell repertoire selection. *Eur J Immunol* **30**:1948-1956
- Fairchild, P.J. and H. Waldmann (2000) Dendritic cells and prospects for transplantation tolerance. *Cur Opin Immunol* **12**(5):528-535
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- Cobbold S.P., K. Nolan, L. Graça, R. Castejon, M. Frewin, S. Humm, E. Adams, S. Thompson, D. Zelenika, A. Paterson, S. Yates, P.J. Fairchild and H. Waldmann (2003)

Regulatory T cells and dendritic cells in transplantation tolerance: molecular markers and mechanisms. *Immunol Rev.* **196**:109-124

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- Graça, L., A. Le Moine, C-Y. Lin, P.J. Fairchild, S.P. Cobbold and H. Waldmann (2004) Donor-specific transplantation tolerance: The paradoxical behavior of $CD4^+CD25^+$ T cells. *Proc. Natl. Acad. Sci. USA* **101**:10122-10126
- Fairchild, P.J., K.F. Nolan, S. Cartland and H. Waldmann (2004) Embryonic stem cells and the challenge of transplantation tolerance. *Trends Immunol.* **25**:465-470
  [Article specially featured in *Life Science Review Magazine*]
- Fairchild, P.J., K.F. Nolan, S. Cartland and H. Waldmann (2004) Embrynoic stem cells: A novel source of dendritic cells for clinical applications. *Int. Immunopharmacol.* In Press.
- Fairchild, P.J., T-C. Chen and H. Waldmann (2005) Prospects for the use of altered peptide ligands in the treatment of allograft rejection. *Cur. Topics Peptide Protein Res.* In Press.

Manuscripts in Preparation

- Yates, S.F., H. Waldmann and P.J. Fairchild (2004) Vitamin D_3 reinforces the tolerogenicity of immature dendritic cells. For submission to *Nat. Med.*
- Chen, T-C., P.J. Fairchild and H. Waldmann (2004) Persistant, danger-free exposure to antigen drives naive T cells to acquire regulatory properties.

Notch signaling is necessary but not sufficient for differentiation of dendritic cells

Pingyan Cheng, Yulia Nefedova, Lucio Miele, Barbara A Osborne, and Dmitry Gabrilovich

The Notch family of receptors plays an important role in regulation of cell differentiation via direct contact between hematopoietic progenitor cells (HPCs) and bone marrow stroma (BMS). However the precise contribution of Notch in dendritic cell (DC) differentiation is controversial. In 2 different experimental systems using Notch-1–null embryonic stem cells and Notch-1–deficient HPCs we have found that Notch-1 is necessary for DC differentiation. However, activation of Notch-1 and Notch-2 with cell-bound Notch ligand did not result in differentiation of mature DCs or macrophages. Instead, it caused accumulation of immature myeloid cells. Removal of feeder cells resulted in rapid differentiation of DCs and macrophages. Addition of interleukin 4 (IL-4) into the culture dramatically increased accumulation of functionally potent DCs. Lipopolysaccharide was not able to reproduce this effect. Thus, these data indicate that Notch signaling prevents differentiation of mature myeloid cells. Instead, it results in accumulation of precursors readily able to differentiate into mature DCs once the Notch signal is stopped (eg, after cell emigration from bone marrow) and in the presence of other additional differentiation signals provided by IL-4. Thus, Notch is required but not sufficient for DC differentiation. (Blood. 2003;102:3980-3988)

Introduction

Dendritic cells (DCs) are professional antigen-presenting cells critically important for induction of immune responses [1-3] Several subsets of these cells including lymphoid, myeloid, and plasmacytoid DCs have been recently identified [4] Differentiation of all DC subtypes is taking place in the bone marrow and it is tightly controlled by a complex network of different soluble growth factors and cytokines and by direct cell-cell contact between hematopoietic progenitor cells (HPCs) and bone marrow stroma (BMS). To date, several growth factors important for DC differentiation have been identified (for a review, see Vuckovic et al[5]) However, the role of direct cell-cell contact in DC differentiation remains unclear.

In recent years Notch signaling emerged as a critical element of the development and maintenance of hematopoiesis The Notch family is a group of highly conserved molecules that are expressed as receptors on the cell surface and directly regulate gene transcription. There is accumulating evidence that the Notch pathway affects survival, proliferation, and cell fate choices at various stages of hematopoietic cell development, including the decisions of HSCs to self-renew or differentiate and of common lymphoid precursors to undergo T- or B-cell differentiation.[6-8] Notch ligands in vertebrates include Jagged-1 and -2, and Delta-1, -2, -3, and -4.[8] Jagged-1 and Delta-1 Notch ligands are expressed on BMS cells. Notch-1 is activated following binding of appropriate ligands on adjacent cells to the extracellular domain of Notch-1 on the surface of HPCs This results in a 2-stage proteolytic cleavage, release, and nuclear translocation of the Notch intracellular domain This domain interacts with a number of cytoplasmic and nuclear proteins, permitting signal transduction through several pathways that include activation of CBF-1/Rbp-Jk transcription factor and *E(spl)/HES* genes, which work as negative regulators of lineage-specific gene expression

An important role of Notch signaling in differentiation of lymphocytes is well established [7-10] The role of Notch signaling in myeloid cell differentiation is much more controversial. In vivo experiments with a conditional knockout of the Notch-1 gene showed no effect on myeloid development.[9,11] Differentiation of plasmacytoid DCs was also not affected [12] Immobilized Notch ligand Delta-1 inhibited differentiation of monocytes into mature macrophages with granulocyte-macrophage colony-stimulating factor (GM-CSF). However, it permitted differentiation into mature DCs.[13] These data might suggest that Notch is not involved in differentiation of myeloid cells in general and DCs in particular. However, a number of studies suggest possible involvement of Notch in myeloid cell differentiation Constitutive expression of the activated intracellular domain of mouse Notch-1 in 32D myeloid progenitors inhibits granulocytic differentiation and permits expansion of undifferentiated cells.[14] Overexpression of intracellular domain of Notch-1 in these cells inhibited differentiation induced by granulocyte colony-stimulating factor (G-CSF), whereas overexpression of corresponding domain of Notch-2 inhibited differentiation induced by GM-CSF [15] Another group has shown that conditional induction of the constitutively active intracellular domain of murine Notch-1 promoted myeloid differentiation via RBP-J transactivation.[16] Recently, Varnum-Finney and colleagues have demonstrated that incubation of murine bone marrow precursors with Notch ligand and growth factors inhibited myeloid differentiation and promoted an increase in the number of precursors capable of short-term lymphoid and myeloid repopulation [17] In a

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different study a soluble form of Notch ligand Jagged-1 was able to induce maturation of monocyte-derived human DCs [18]

Taken together these data depict a rather convoluted picture of the possible involvement of Notch in DC differentiation. In this study we have tried to clarify the potential role of Notch signaling in myeloid cell differentiation in general, and DCs, in particular.

Materials and methods

Mice

Female Swiss mice (6-8 weeks old) were purchased from Charles River Laboratories (Wilmington, MA) Female BALB/c and C57BL/6 mice (6-8 weeks old) were obtained from Harlan (Indianapolis, IN) All mice were housed in specific pathogen-free units of the Division of Comparative Medicine at University of South Florida (Tampa, FL). Notch-1 antisense transgenic (Notch-AS-Tg) mice were described elsewhere [19] Briefly, mice were generated using a Notch-1 antisense construct expressed under the control of the mouse mammary tumor virus long terminal repeat promoter The genetic background of the founders was C57BL/6:SJL F1, and the mice were then back-crossed for 4 generations with C57BL/6 mice Hemizygous transgenic mice were then bred to each other, selecting homozygous transgenic and negative mice until 2 syngeneic strains derived from the same original litter were obtained: one homozygous antisense Notch-1 transgenic (Notch-AS-Tg) and one nontransgenic (control) The specificity of Notch-1 down-regulation was confirmed by Western blotting [19]

Reagents

The following antibody-producing hybridomas were purchased from the American Type Culture Collection (Manassas, VA) and used as supernatants: anti-CD4 (TIB-207); anti-CD8 (TIB-210); and anti-MHC II (TIB-120) Anti-TER-119. B220, Gr-1 (anti-Ly-6G), IA^b, IA^q, CD11b, CD86 (B7-2), CD45, CD11c, CD34, Sca-1, c-Kit, and isotype control antibodies were obtained from PharMingen (San Diego, CA) Anti-F4/80 antibody was purchased from Serotec (Raleigh, NC); antiphycoerythrin (anti-PE) or streptavidin microbeads were from Miltenyl Biotec (Auburn, CA) Antibodies against Notch-1 (bTAN20) and Notch-2 (C651 6DbHN) were purchased from Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, IA); anti–Notch-3 and anti–Notch-4 were from Santa Cruz Biotechnology (Santa Cruz, CA) Low-Tox rabbit complement, and Lympholyte M were purchased from Cedarlane Laboratories (Hornby, ON, Canada) Recombinant human FLT-3 ligand (FL), recombinant murine GM-CSF, G-CSF, macrophage colony-stimulating factor (M-CSF), interleukin 4 (IL-4), IL-3, and tumor necrosis factor α (TNF-α) were obtained from Research Diagnostics (Flanders, NJ), lipopolysaccharide (LPS) from Sigma (St Louis, MO), and poly(dI-dC) from Pharmacia (Piscataway, NJ)

Preparation of enriched HPCs

Bone marrow cells were harvested from the femurs and tibias of mice and enriched for HPCs by depletion of lineage-specific cells as described earlier [19] Briefly, bone marrow cells were incubated with mixture of antibodies (TIB-207, TIB-210, TIB-120, anti–TER-119, anti–Gr-1, anti-B220) for 30 minutes on ice, washed, and treated with complement for 1 hour at 37°C Dead cells were then removed by centrifugation over a Lympholyte M gradient The resulting fraction contained less than 20% of lineage-positive cells as detected by flow cytometry

NIH-3T3-Jagged-1–expressing cell line

NIH-3T3-Jagged-1–expressing cell line (3T3-Jagged) was a gift from Irwin D Bernstein (Fred Hutchinson Cancer Center, Seattle, WA) and the generation of the cell line was described previously [20] A control fibroblast cell line NIH-3T3-vector (3T3-MSCV) was generated by infecting parental NIH-3T3 cells with empty retroviral vector MSCV, and selected with 1 mg/mL G418

Generation of DCs from HPCs

DCs were generated from HPCs using FL as described earlier with minor modification [21] Briefly, 5×10^5 HPCs per well of 24-well plates were cultured for 10 days in 2 mL complete medium supplemented with 200 ng/mL FL and 10% of conditioned medium from splenocytes On day 9, 1 μg/mL LPS was added, and cells were cultured for additional 24 hours, collected, washed, and used for further analysis For generation of DCs and other myeloid cell on fibroblasts control and Jagged 3T3 fibroblasts were irradiated with 25 Gy and cultured overnight in 24-well plates (1.5×10^5 cells/well) Half million HPCs were placed into each well in 2 mL 10% fetal bovine serum (FBS) RPMI supplemented with 20 ng/mL GM-CSF or M-CSF for 7 or 10 days In some experiments to generate DCs, IL-4 (10 ng/mL) was also added Every 3 days, cells were transferred onto new fibroblasts and medium was replaced with growth factors. For DC activation, TNF-α (5 ng/mL) was added 48 hours prior to cell analysis On day 7 or day 10, all cells were collected and analyzed for DC, macrophage, or granulocyte phenotype

DC differentiation of Notch-1$^{-/-}$ ES cells

Differentiation of DCs from embryonic stem (ES) cells was performed as described earlier[22] with modifications Notch-1$^{+/+}$ and Notch-1$^{-/-}$ ES cell lines were kind gifts from Dr Conlon (Case Western Reserve University, Cleveland, OH) They were made in R1-S3 ES cell line and were described in detail elsewhere [23] ES cells were grown on gelatinized plates in complete knockout Dulbecco modified Eagle medium (DMEM) supplemented with 15% ES-qualified FBS (Gibco, Rockville, MD), 0.1 mM nonessential amino acid, 100 μM 2-mecaptoethenol, 2 mM L-glutamine, and leukocyte inhibitory factor (LIF; 1000 U) To induce formation of embryonic bodies hematopoietic cell ES cells were transferred on bacterial-grade Petri dishes with LIF-free knockout DMEM supplemented with 15% ES-qualified FBS and 100 μM 2-mercaptothenol and incubated for 14 days Medium was changed every other day On day 14, one embryonic body was transferred to each well of a 24-well plate and incubated in 2 mL/well 15% FBS RPMI medium with 20 ng/mL GM-CSF and 10 ng/mL IL-3 for 30 days Half of the medium was changed every 3 days TNF-α (5 ng/mL) was added for the last 5 days before DC analysis

Isolation of CD45$^+$ and Gr-1$^+$ cells from HPCs cocultured with 3T3 fibroblasts

CD45$^+$ cell or Gr-1$^+$ cell isolation was performed using MiniMACS microbeads according to the manufacturer's protocol (Miltenyi Biotec). After incubation with fibroblasts, cells were collected, washed twice with phosphate-buffered saline (PBS), and incubated with either antimouse CD45 PE-conjugated antibody or antimouse Gr-1 biotinylated antibody on ice for 10 minutes Then, cells were washed and incubated with either anti-PE microbeads or streptavidin microbeads on ice for 10 minutes After washing, cells were separated on MACS columns and used in experiments Purity for CD45$^+$ or Gr-1$^+$ cells was more than 93% in all samples.

Analysis of the phenotype of myeloid cells

The phenotype of DCs and myeloid progenitors was analyzed on FACSCalibur flow cytometer (BD Biosciences, Mountain View, CA) using a CellQuest program (Becton Dickinson, Mountain View, CA) A combination of antibodies conjugated with different fluorochromes was used in multicolor analysis

Allogeneic MLR

Allogeneic mixed leukocyte reaction (MLR) was used to determine DC function Briefly, DCs were sorted using FACSVantage SE cell sorter (BD Biosciences) and were cultured for 4 days in triplicate in U-bottom 96-well plates with lymph node cells (10^5/well) obtained from allogeneic BALB/c mice Then, 1 μCi (0.037 MBq) thymidine 24 was added to each well 18 hours before cell harvest T-cell proliferation was measured by [^{3}H]-thymidine incorporation using a liquid scintillation counter (Packard Instrument, Meriden, CT)

EMSA

Electrophoretic mobility shift assay (EMSA) was performed as previously described[24] Briefly, double-stranded oligonucleotides containing the specific binding site for CBF-1 were made by annealing the appropriate single-stranded oligonucleotides at 65°C for 10 minutes The probes were labeled with α^{32}P deoxyadenosine-5' triphosphate (dATP) (6000 μCi/mmol [222 0 MBq]; Amersham Life Sciences, Arlington Heights, IL) using Klenow DNA polymerase The 2 probes used were wild-type 5'-TGGTGTAAACACG-CCGTGGGAAAAAATTTA-3') and mutant 5'-TGGTGTAAACACGC-CGTTGGAAAAAATTTA-3')

HPCs were cultured overnight with 20 ng/mL GM-CSF, washed, and starved for 2 hours in serum-free medium Two million HPCs per well of 24-well plates were placed on the irradiated 3T3-control or 3T3-Jagged-1 fibroblasts After coculture CD45$^+$ cells were isolated and EMSA was performed using nuclear extracts[25] Nuclear extract (5 μg) was incubated with labeled probe in binding buffer containing 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid), 5% glycerol, 0 2 mM EDTA (ethylenediaminetetraacetic acid), 1 mM dithiothreitol (DTT), 5 mM $MgCl_2$, and 4 μg poly(dI-dC) Specific competition assays were performed with a 50-fold excess of unlabeled probes For CBF-1–binding blockage, 4 μg of monoclonal antibodies (mAbs) against Notch-1 or Notch-2 were incubated with nuclear proteins for 30 minutes on ice prior to addition of α^{32}P-labeled CBF-1 probe The samples were separated on 4% polyacrylamide gels, and bands were visualized by overnight exposure to x-ray films (Fuji, Stamford, CT) at −70°C

Western blot assay

Cells were lysed for 30 minutes on ice in lysis buffer (50 mM Tris [tris(hydroxymethyl)aminomethane]–HCl, pH 7 5, 150 mM NaCl, 0 5% Nonidet P-40, 1% Triton X-100, 0 5% sodium deoxycholate, 100 mM Na_3VO_4, 20 mM NaF, 1 mM phenylmethylsulfonyl fluoride [PMSF], 10 μg/mL aprotinin, 10 μg/mL leupeptin, 10 μg/mL pepstatin) Samples were subjected to electrophoresis on 10% sodium dodecyl sulfate–polyacrylamide gels followed by transfer to a polyvinylidene difluoride membrane and probing with specific antibody The bands were visualized by an enhanced chemiluminescence (ECL) detection kit (Amersham Life Sciences)

HPC proliferation on fibroblasts with cytokines

Irradiated fibroblasts (5 × 10^4/well) were cultured overnight in flat-bottom 96-well plates HPCs (10^5/well) were plated on the fibroblasts for 2 or 4 days in triplicates with 20 ng/mL GM-CSF or GM-CSF and 10 ng/mL IL-4 Then 1 μCi (0 037 MBq) [^{3}H]-thymidine, was added to each well 18 hours before cell harvest HPC proliferation was measured by [^{3}H]-thymidine incorporation using a liquid scintillation counter

Expression of GM-CSF receptor, IL-4 receptor, and Flt-3–specific mRNA

HPCs were cultured with GM-CSF on 3T3-MSCV or 3T3-Jagged fibroblasts for 48 hours or 72 hours CD45$^+$ cells were isolated and the total RNA was extracted using the TRIZOL reagent (Life Technologies) Traces of DNA were removed by treatment with DNase I The cDNA was synthesized from 1 μg total RNA using random hexamers and Superscript II reverse transcriptase (Life Technologies) Samples were subjected to initial denaturation at 94°C for 3 minutes and 28 cycles of polymerase chain reaction (PCR; 94°C for 30 seconds, 58°C for 30 seconds, 72°C for 45 seconds) with final extension for 7 minutes at 72°C The number of cycles was selected after preliminary experiments to avoid saturation of the PCR products

The PCR primer pairs used in this study: *GM-CSFRα*[25]: forward, 5'-GCGGGCGACACGAGGATGAAGCAC; reverse, 5'-CTAGGGCTG-CAGGAGGTCCTTCCT *IL-4R*[26]: forward, 5'-GCTAGTTGTCATCCT-GCTC; reverse, 5'-GTGATGTGGACTTGGACTC; *Flt-3*: forward, 5'-GGCTCTGTCTCCCCTTCATTG; reverse, 5'-GCCCCCAGCAGATTCACG; hprt: forward, 5'-GATTCAACTTGCGCTCATCTTAGGC; reverse, 5'-GTT-GGATACAGGCCAGACTTTGTTG

The PCR products were visualized on 1% agarose gel The sizes of PCR products were 263 bp, 352 bp, 393 bp, and 164 bp, respectively PCR products were transferred in an alkaline transfer buffer (0 4 N NaOH, 1 M NaCl) onto Hybond N$^+$ nylon transfer membranes (Amersham, Highland Park, IL) and probed with ^{32}P-labeled oligonucleotide probes: *GM-CSFRα*, 5'-TGTCCTCAGCCTCGAGAGGATG-3'; *IL-4R*, 5'-TGCCAAACGTC-CTCACAGC-3'; *Flt-3*, 5'-CAGGTGGCGGTGAAGATGC-3'; hprt, 5'-GTTGTTGGATATGCCTTGAC-3'

Statistical analysis

Statistical analysis was performed using parametric methods and JMP statistical software (SAS Institute, Cary, NC)

Results

Notch-1 is necessary for differentiation of DCs

To address the question whether Notch signaling is necessary for DC differentiation we generated DCs from control (Notch-1$^{+/+}$) and Notch-1$^{-/-}$ ES cells using a recently established experimental system described in "Materials and methods" Lack of Notch-1 protein in Notch-1$^{-/-}$ cells was confirmed by Western blotting (Figure 1A) This protocol uses GM-CSF and IL-3 and promotes differentiation of immature DCs To activate DCs, 5 ng/mL TNF-α was added for the last 5 days before the analysis Around 40% of embryonic bodies from both Notch-1$^{+/-}$ and Notch-1$^{-/-}$ ES cells were able to produce clusters of loosely attached cells Analysis of the cell phenotype demonstrated that more than 90% of the cells generated from control ES cells expressed the CD11c marker of DCs Both myeloid-related (CD11c$^+$CD11b$^+$) and lymphoid-related (CD11c$^+$CD11b$^-$) DC populations were present (Figure 1B), whereas plasmacytoid DCs (Gr-1$^+$CD11c$^+$B220$^+$) were not detectable (data not shown) More than 75% of these DCs expressed major histocompatibility complex (MHC) class II and almost 70% expressed B7-2 molecules (Figure 1B) These cells were potent stimulators in allogeneic MLRs (Figure 1C) These data were consistent with a previously published observation[22] and confirmed the fact that this experimental protocol allows for generation of mature DCs from ES cells Differentiation of DCs from Notch-1$^{-/-}$ ES cells was significantly impaired Although the total number of cells generated from individual Notch-1$^{+/+}$ and Notch-1$^{-/-}$ embryonic bodies was the same, the proportion of both myeloid and lymphoid DCs was reduced more than 2-fold (Figure 1B) The proportion of cells expressing MHC class II and costimulatory B7-2 (CD86) molecules was also more than 2-fold lower than in cells generated from control ES cells (Figure 1B) To test whether these changes in phenotype were associated with functional changes, these cells were used as stimulator in allogeneic MLRs, the function specifically attributed to DCs The ability of cells generated from Notch-1$^{-/-}$ ES cells to stimulate allogeneic T cells was significantly lower than in their control counterparts, which reflects the decreased presence of functionally competent DCs (Figure 1C) These data demonstrated that Notch-1 is critically important for differentiation of DCs from ES cells

As shown in Figure 1 a large number of cells generated from Notch-1$^{-/-}$ ES cells lacked expression of DC-specific markers Using flow cytometry and antibodies specific for different cell lineages we analyzed the population of CD11c$^-$ cells About one third (32 4% ± 4.5%) of CD11c$^-$ cells generated from Notch-1$^{+/+}$ ES cells were CD45$^+$ hematopoietic cells C-kit$^+$Sca-1$^+$ hematopoietic stem cells represented 3.5% ± 0 3% and Gr-1$^+$ CD11b$^+$ immature myeloid cells 3 1% ± 1.1% of CD11c$^-$ cells The rest of

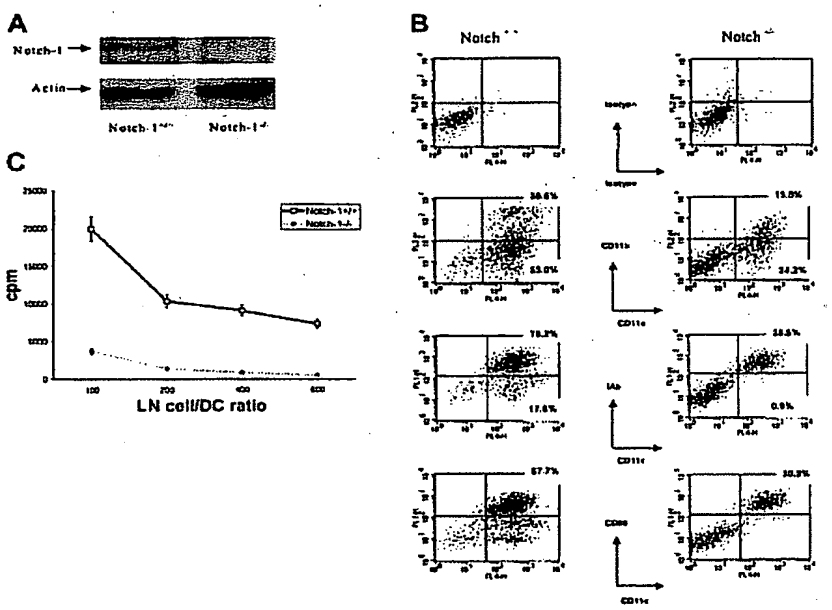

Figure 1. Notch-1$^{-/-}$ ES cells have reduced capacity to differentiate into DCs. (A) Whole cell lysates were prepared from Notch-1$^{+/+}$ and Notch-1$^{-/-}$ ES cells, and the presence of Notch-1 protein was determined by Western blotting as described in "Materials and methods." (B) Embryonic bodies were cultured with GM-CSF and IL-3 for 25 days, followed by a 5-day incubation with TNF-α. Cells were labeled with allophycocyanin (APC)–conjugated anti-CD11c, PE-conjugated anti-CD11b, and fluorescein isothiocyanate (FITC)–conjugated anti-IA^b or anti–B7-2 antibodies. Three experiments with the same results were performed. (C) DCs were generated from ES cells as described, irradiated at 150 Gy, and cultured with lymph node (LN) cells from control allogeneic BALB/c mice at different ratios. Cell proliferation was measured in triplicates as described in "Materials and methods." Values are the average ± SE from 2 experiments.

the CD45$^+$ cells were Gr-1$^+$CD11b$^-$ granulocytes and CD11b$^+$F4/80$^+$ macrophages. In sharp contrast, only 2.0% ± 0.5% of CD11c$^-$ cells generated from Notch-1$^{-/-}$ ES cells expressed CD45. About 1% of the cells were c-kit$^+$Sca-1$^+$. Other myeloid cells represented less than 1% of CD11c$^-$ cells (data not shown).

Next, we checked the expression of Notch proteins during DC differentiation from ES cells. Notch-1 was expressed in wild-type ES cells and embryonic bodies and disappeared when cells started differentiation into DCs (Figure 2). Notch-2 expression in Notch-1$^{-/-}$ ES cells was substantially higher than in Notch-1$^{+/+}$ ES cells. This may represent a compensatory mechanism, which occurs in Notch-1–deficient cells. Notch-2 expression became undetectable during cell differentiation into embryonic bodies (next 2 weeks) and then slightly increased when cells started differentiation into DCs (day 20; Figure 2). Notch-3 was expressed in both wild-type and Notch-1$^{-/-}$ ES cells, but not at later stages of cell differentiation (Figure 2). Notch-4 was undetectable at all stages of cell differentiation (data not shown).

To verify the effect of Notch-1 deficit on DC differentiation we used the other experimental system that used FL and allowed for

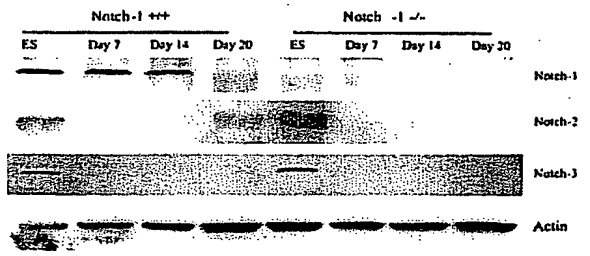

Figure 2. Notch family members expressed on different stages of ES cell differentiation. Whole cell lysates were prepared from ES cells or cells at different stages of differentiation. Western blot was performed using anti–Notch-1, -2, -3, and β-actin antibodies as described in "Materials and methods." ES indicates embryonic stem cells cultured with LIF; day 7 and day 14, embryonic body development from ES cells for 14 days; day 20, ES cell DC generation for 6 days with GM-CSF and IL-3 after 14-day embryonic body development.

generation of both myeloid-related and lymphoid-related DCs from HPCs.[21] Enriched HPCs were isolated from bone marrow of control or Notch-deficient (Notch-As-Tg) mice. As we reported earlier, the levels of Notch-1 protein in HPCs from Notch-As-Tg mice was 2-fold lower than that in HPCs from control mice.[19] Cells were cultured with FL as described in "Materials and methods." To activate DCs, 1 μg/mL LPS was added to the culture 24 hours prior to analysis. No differences in the total number of cells (data not shown) or in the proportion of populations of CD11c$^+$CD11b$^+$ or CD11c$^+$CD11b$^-$ DCs were found between cells isolated from these 2 groups of mice (Figure 3A). These data might indicate that the decrease in Notch-1 protein in HPCs was not sufficient to cause a significant decrease in DC differentiation as was the case in Notch-1–null ES cells. However, when we evaluated the expression of markers attributed to mature DCs, dramatic differences were found within both populations of cells. In DCs generated from HPCs of Notch-As-Tg mice the expression of MHC class II, B7-2, and CD40 molecules was significantly reduced. It manifested in a significant decrease in the proportion of cells positive for MHC class II, B7-2, and CD40 (Figure 3B) and in more than a 2-fold decrease in the intensity of fluorescence (data not shown). The defect in differentiation of both populations of DCs was confirmed in a functional test. DCs generated from HPCs as described were sorted into 2 populations: CD11c$^+$CD11b$^+$ myeloid-related and CD11c$^+$CD11b$^-$ lymphoid-related DCs, and used in allogeneic MLRs. Both populations of DCs generated from Notch-1-AS-Tg mice had a significantly lower ability to stimulate allogeneic T-cell proliferation than the cells generated from control mice (Figure 3C-D). Less than 2% of DCs generated with FL expressed markers of plasmacytoid DCs (Gr-1$^+$CD11c$^+$B220$^+$). No differences were observed between control and Notch-As-Tg mice (data not shown).

These data demonstrate that even a partial decrease of Notch-1 protein in HPCs inhibits differentiation of mature, functionally potent DCs. Taken together, these data indicate that Notch-1 is necessary for normal differentiation of both myeloid and lymphoid DCs.

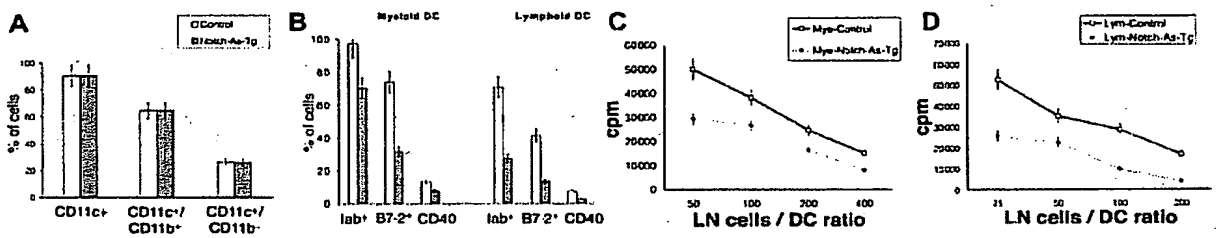

Figure 3. HPCs from Notch-1–deficient mice had reduced capacity to differentiate into DCs. (A-B) HPCs were isolated from control or Notch-1–deficient Notch-AS-Tg mice and incubated with 200 ng/mL FL and 10% splenocyte-conditioned medium for 10 days. LPS (1 μg/mL) was added 24 hours before cell phenotype analysis. Cells were labeled with APC-conjugated anti-CD11c, PE-conjugated anti-CD11b, and FITC-conjugated anti-IA^b, anti–B7-2, or CD40 antibodies. Proportions of IA^{b+}, B7-2^+, or $CD40^+$ cells were calculated within the populations of $CD11c^+CD11b^+$ myeloid DCs and $CD11c^+CD11b^-$ lymphoid DCs. Results of 3 performed experiments are shown. Differences between control and Notch-As-Tg mice within populations of lymphoid and myeloid DCs were statistically significant ($P < .05$). (C-D) DCs were generated from HPCs as described. The same 2 populations of DCs were sorted using FACSVantage SE cell sorter, irradiated at 150 Gy, and cultured with lymph node (LN) cells isolated from control allogeneic BALB/c mice. Cell proliferation was measured in triplicate. Values are the average ± SE from 3 experiments. Differences between values in control and Notch-AS-Tg mice were statistically significant at all LN cell/DC ratios ($P < .05$).

Activation of Notch in HPCs by fibroblasts expressing Jagged-1

These data indicated that deficit of Notch-1 in stem cells or HPCs resulted in impaired differentiation of DCs. Next we asked whether ligand-mediated activation of Notch is sufficient for DC differentiation. HPCs isolated from control mice were cultured on a monolayer of fibroblasts transfected with either MSCV vector (control) or Notch ligand Jagged-1 expression vector (Jagged-1). Different growth factors were used to generate different populations of myeloid cells. HPCs from Swiss mice were used in these experiments to match the haplotype of fibroblasts.

First, we have determined whether these conditions result in Notch activation. Notch-1 and Notch-2 are both expressed on mouse HPCs and can bind to the ligand Jagged-1. After binding, the intercellular domain of Notch is released and translocates to the nuclei where it binds to CBF-1 transcription factor and releases it from the repressor complex with histone deacetylase (HADC) and nuclear corepressor (CoR). CBF-1 in complex with Notch acts as a transcription activator.

To evaluate Notch activation by Jagged-1 we performed EMSA with CBF-1–specific oligonucleotides. HPCs were cultured for 5 hours on control or Jagged-1 fibroblasts, $CD45^+$ cells were isolated, and nuclear proteins were extracted and used in EMSA. Jagged-1 induced substantial activation of CBF-1, which was manifested in increased specific binding to DNA compared with control (Figure 4, lanes 2-3). To further determine whether Notch-1 and Notch-2 are equally involved in the Jagged-1–mediated activation, we used antibodies against Notch-1 or Notch-2. Nuclear extracts were pretreated with these antibodies and then used in EMSA. Specific binding of this antibody should prevent a Notch/CBF-1 complex from binding to DNA. In our experiments control antibody (TIB-120 against mouse $I\text{-}A^{d,k}$) did not affect CBF-1 binding (Figure 4, lane 4), whereas anti–Notch-1 antibody inhibited it (Figure 4, lane 5). Similarly, inhibition of CBF-1–binding activity was observed with antibody against Notch-2 (Figure 4, lane 6). However, the effect was slightly lower than that of anti–Notch-1 antibody. Combination of 2 antibodies completely blocked the CBF-1 binding (lane 7). These results demonstrated that in HPC Jagged-1 activated both Notch-1 and Notch-2.

Effect of Notch activation on DC differentiation from mouse HPCs

Bone marrow cells enriched for HPCs were placed in 24-well plates containing a monolayer of either control or Jagged-1 fibroblasts and cultured with FL for 10 days. $CD45^+$ leukocytes were analyzed using flow cytometry. To our surprise incubation on Jagged-1 fibroblasts did not affect the total number of cells (data not shown) but generated significantly lower proportion of DCs (Figure 5A). Because generation of DCs depends on several different growth factors, we tested the effect of Notch activation in the presence of the other cytokine able to support DC differentiation, specifically GM-CSF. After a 7-day culture on control or Jagged-1 fibroblasts with GM-CSF alone the number of $CD45^+$ cells was equal (data not shown). The cell proliferation assay confirmed that there was no difference in proliferation between these 2 groups (Figure 5B). As in case of FL, HPCs grown on NIH 3T3-Jagged-1 fibroblasts generated fewer DCs than HPCs grown on control fibroblasts. Instead, accumulation of cells with the phenotype of immature myeloid cells (ImCs; $Gr\text{-}1^+CD11b^+$) was observed (Figure 5C). To test whether this phenomenon is limited to DC differentiation M-CSF was used to differentiate macrophages from HPCs. The total number of $CD45^+$ cells generated in the presence of M-CSF was also not different between the groups (data not shown). However, as in case of GM-CSF a substantially higher proportion of ImCs was generated from HPCs grown on Jagged-1 than on control fibroblasts (Figure 5D). The proportion of mature $F4/80^+$ macrophages was slightly decreased (Figure 5D). It

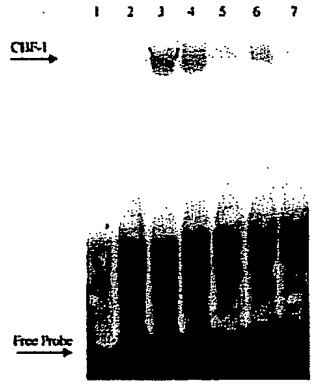

Figure 4. Activation of Notch-1 and Notch-2 by ligation with Jagged-1. HPCs were placed on 3T3-MSCV or 3T3-Jagged-1 fibroblasts for 5 hours; $CD45^+$ cells were isolated and nuclear protein was extracted. CBF-1 binding to DNA was determined by EMSA. For blockade of CBF-1 binding 2 μg/lane nuclear protein was preincubated with specific antibodies against Notch-1 or Notch-1, for 30 minutes on ice prior to incubation with a $\alpha\text{-}^{32}P$ probe. Fifty-fold excess of unlabeled "cold" wild-type probe competitor in sample from cells incubated with Jagged-1 fibroblasts (lane 1); samples from cells cocultured with control (lane 2) and Jagged-1 fibroblasts (lane 3); blockade of CBF-1–binding activity with control antibody against mouse $I\text{-}A^{d,k}$ (lane 4), antibody against Notch-1 (lane 5), antibody against Notch-2 (lane 6), and combination of antibodies against Notch-1 and Notch-2 (lane 7). Two experiments with the same results were performed.

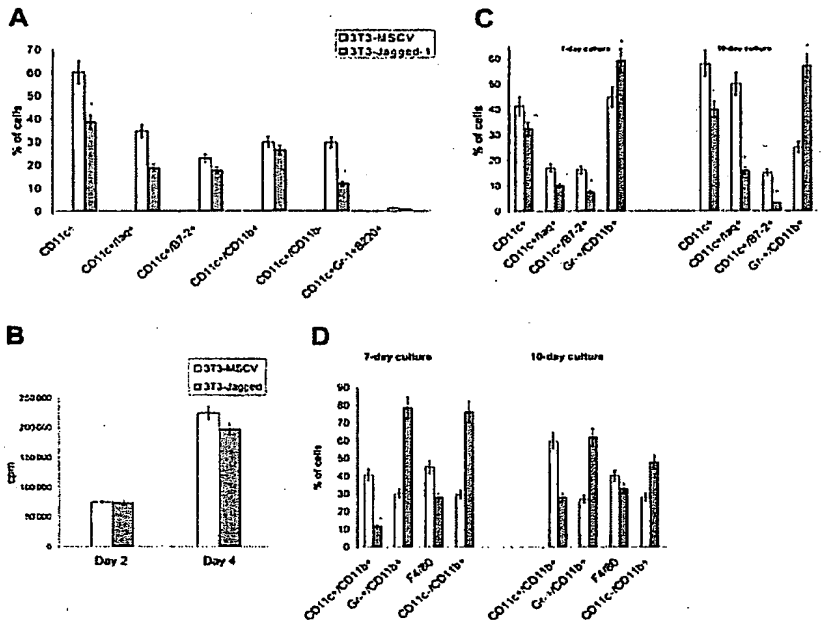

Figure 5 Accumulation of immature myeloid cells after activation of Notch signaling in HPCs. (A) HPCs were cultured on fibroblasts in 24-well plate with FL and supernatants from control splenocytes for 10 days as described in "Materials and methods." Cells were then labeled with APC-conjugated anti-CD11c or anti-Gr-1 antibodies, PE-conjugated anti-CD45 antibody, peridinin chlorophyll protein (PerCP)–conjugated anti-B220 antibody, and FITC-conjugated anti-IA^q, anti–B7-2, CD11b, anti-F4/80 or Gr-1 antibodies and analyzed on a FACSCalibur flow cytometer. Only $CD45^+$ cells were evaluated for cell phenotype. Values are the average ± SE from 3 experiments. (B) HPCs were plated on fibroblasts in 96-well plate and incubated for 2 or 4 days with 20 ng/mL GM-CSF. Cell proliferation was measured by [^{3}H]-thymidine incorporation. (C-D) HPCs were cultured on fibroblasts in 24-well plate with GM-CSF (C) or M-CSF (D) for 7 or 10 days. In GM-CSF culture (C), 5 ng/mL TNF-α was added 48 hours before cell phenotype analysis. Cells were labeled with APC-conjugated anti-CD11c or anti–Gr-1 antibodies, PE-conjugated anti-CD45, and FITC-conjugated anti-IA^q, anti–B7-2, CD11b, or anti-F4/80 antibodies and analyzed on a FACSCalibur flow cytometer. Only $CD45^+$ cells were evaluated for cell phenotype. Values are the average ± SE from 3 experiments. Asterisk indicates statistically significant differences between cells treated with 3T3-MSCV and 3T3-Jagged-1 fibroblasts.

was possible that in an experimental system containing fibroblasts myeloid cell differentiation might be delayed, which would explain the decreased proportion of mature DCs and macrophages. To test this possibility, we extended culture period from 7 days to 10 days. As shown in Figure 5C-D, an extended 10-day culture with GM-CSF or M-CSF did not result in generation of more mature DCs or macrophages and did not decrease the proportion of ImCs. On the contrary, extension of incubation resulted in decreased proportion of DCs (Figure 5C). Thus, our results indicate that Notch activation through cell-associated Jagged-1 ligation promotes accumulation of ImCs rather than differentiation of mature macrophages or DCs.

Withdrawal of Notch signaling permits differentiation of ImCs

We analyzed the phenotype of ImCs accumulated as a result of Notch activation. HPCs were cultured for 7 days on control or Jagged-1 fibroblasts with GM-CSF or M-CSF. $Gr\text{-}1^+$ cells were isolated using a magnetic bead separation technique. The resulting population had more than 93% $Gr\text{-}1^+CD11b^+$ cells. These cells were then labeled with antibodies against markers specific for HPCs and stem cells: CD34, c-kit ligand, and Sca-1. $Gr\text{-}1^+$ cells generated on Jagged-1 fibroblasts had 3 to 5 times higher proportion of HPCs than $Gr\text{-}1^+$ generated on control fibroblasts (Figure 6A). $CD34^+$ cells represented almost 25% of all $Gr\text{-}1^+$ cells generated on Jagged-1 fibroblasts, whereas their proportion in $Gr\text{-}1^+$ cells generated on control fibroblasts was less than 5%.

Next, we asked whether Notch activation through Jagged-1 is necessary for maintaining the phenotype of immature cells. Enriched HPCs were cultured for 5 days with GM-CSF on control or Jagged-1 fibroblasts; $CD45^+$ myeloid cells were isolated and transferred into new plates. These plates either contained control fibroblasts or were without any fibroblasts. Cells were cultured for additional 5 days with either GM-CSF or M-CSF. In culture with GM-CSF, 1 μg LPS was added 24 hours prior to cell analysis. In total, cells were cultured for 10 days, exactly the same time as in experiments shown in Figure 5B-C. However, the results of these experiments were dramatically different. The 5-day culture of the cells without Jagged-1 fibroblasts resulted in a significant decrease in the proportion of $Gr\text{-}1^+CD11b^+$ ImCs and accumulation of relatively mature DCs and macrophages (compare Figure 5B-C and

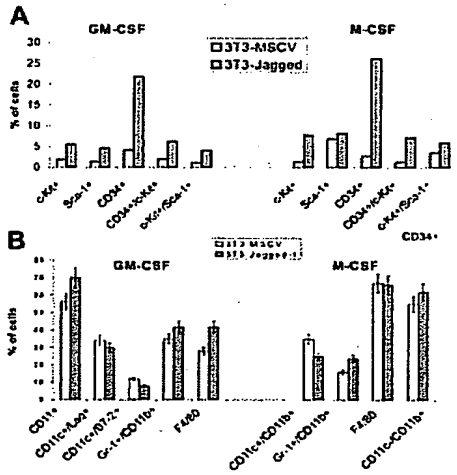

Figure 6. Withdrawal of Notch signaling permitted $Gr\text{-}1^+/Mac1^+$ ImC differentiation. (A) HPCs were cultured on fibroblasts with either GM-CSF or M-CSF for 7 days. $Gr\text{-}1^+$ cells were isolated and stained with antibodies against markers of hematopoietic progenitors: APC-conjugated c-Kit, FITC-conjugated Sca-1, and PE-conjugated CD34 and analyzed on a FACSCalibur flow cytometer. (B) HPCs were cultured on fibroblasts with GM-CSF for 5 days; $CD45^+$ cells were isolated and further cultured without fibroblasts for additional 5 days with either GM-CSF or M-CSF. In the case of GM-CSF culture, 5 ng/mL TNF-α was added 48 hours before cell phenotype analysis. Cells were labeled with APC-conjugated anti-CD11c or anti–Gr-1 antibodies, PE-conjugated anti-CD11b, and FITC-conjugated anti-IA^q, anti–B7-2, or anti-F4/80 antibodies and analyzed on a FACSCalibur flow cytometer. Values are the average ± SE from 3 experiments. Similar results were obtained when cells were cultured for an additional 5 days on control fibroblasts.

Figure 6B) Previously observed differences between the cells grown on Jagged-1 and control fibroblasts (Figure 5B-C) now completely disappeared (Figure 6B). Taken together, these data demonstrate that Notch signaling mediated by Jagged-1 results in accumulation of HPCs and ImCs and prevents differentiation of mature DCs and macrophages Withdrawal of this signal allows for terminal differentiation of DCs and macrophages

Activation of Notch by Jagged-1 ligation together with IL-4 enhances DC differentiation from HPCs

IL-4 is known to provide strong signals supporting selective differentiation of DCs from HPCs We evaluated Jagged-1 effects on DC differentiation in the presence of IL-4. HPCs were cultured on Jagged-1 or control fibroblasts for 7 days with GM-CSF and IL-4. After that time the number of CD45$^+$ cells in these 2 groups was very similar ($1.62 \pm 0.25 \times 10^6$/well for control and $1.60 \pm 0.26 \times 10^6$/well for 3T3-Jagged-1 fibroblasts, $P > 1$) No differences were also seen in cell proliferation assay (Figure 7A) However, HPCs cultured on Jagged-1 fibroblasts produced 2-fold more DCs than HPCs cultured on control fibroblasts (Figure 7B)

Cells generated on 3T3-Jagged-1 fibroblasts had a significantly higher ability to stimulate allogeneic T-cell proliferation in MLRs, which is indicative of higher proportion of DCs (Figure 7C) These data suggest that IL-4 may provide the necessary signal to promote the complete differentiation of immature cells accumulated as the result of the effect of Jagged-1 To test this hypothesis we used another system to generate DC from HPC using FL. The experimental system with FL provides conditions for generation of both lymphoid and myeloid DCs (Figure 7D) Incubation of HPCs on Jagged-1 fibroblasts did not affect the total number of cells (data not shown) but generated a significantly higher proportion of DCs (Figure 7D) This was especially evident in the population of myeloid-related DCs A significant increase was also observed in the proportion of plasmacytoid DCs (from 0 8% to 4 7%, $P < 05$; Figure 7D)

IL-4 can interfere with Notch signaling and thus affect cell differentiation, or alternatively it can provide a strong differentiation signal independent on Notch. To test the direct effect of IL-4 on Notch activation HPCs were cultured with GM-CSF alone or with GM-CSF and IL-4 on control or Jagged-1 fibroblasts On day 5, CD45$^+$ cells were isolated and nuclear protein was used for CBF-1–binding activity The presence of IL-4 did not affect activation of Notch by Jagged-1 (Figure 7E), which suggested that IL-4 did not affect Notch activation during differentiation of DCs.

Notch activation does not affect cytokine receptors and does not activate DCs

Notch activation in HPCs could result in up-regulation of the receptors for cytokines critically important for myelopoiesis. This could affect accumulation of ImCs or DCs. To address this possibility we evaluated the expression of the genes encoding receptors for GM-CSF, FL, and IL-4 Notch activation in HPCs did not affect the expression of any of the tested genes (Figure 8).

To investigate whether proinflammatory signals can provide effects similar to IL-4 on DC differentiation, enriched HPCs were incubated on control or Jagged-1 fibroblasts for 5 days with GM-CSF and with or without LPS (1 μg/mL) HPCs incubated on Jagged-1 fibroblasts generated a lower proportion of DCs than the cells incubated on control fibroblasts LPS did not change this effect (data not shown). Similar results were obtained when cytosine-phosphorothiolated guanine-containing oligonucleotide

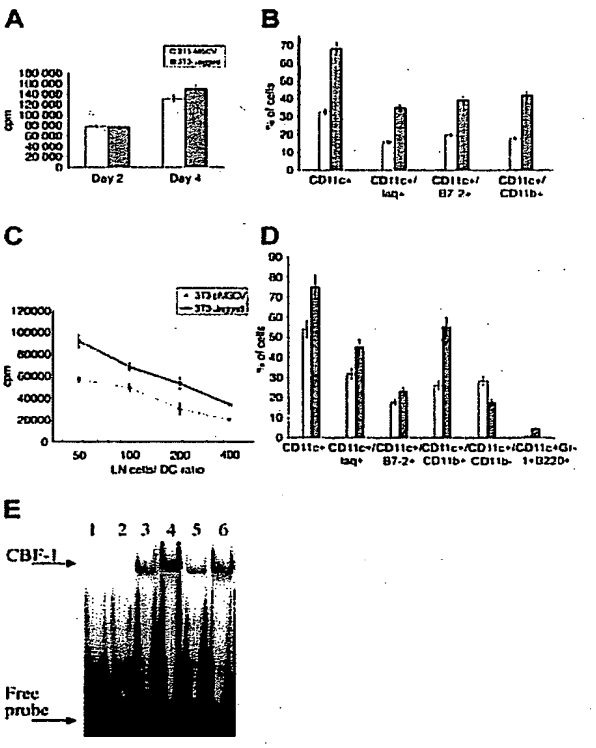

Figure 7 IL-4 enhances DC differentiation during Notch activation (A) HPCs were plated on the fibroblasts in triplicate on 96-well plate with GM-CSF and IL-4 and cultured for 2 or 4 days Cell proliferation was measured by [^{3}H]-thymidine incorporation (B) HPCs were cultured on control or Jagged-1 fibroblasts with GM-CSF and IL-4 for 7 days. Cells were labeled with APC-conjugated anti-CD11c. PE-conjugated anti-CD45. FITC-conjugated anti-IA^q, anti–B7-2. Gr-1 or anti-CD11b antibodies. and PerCP-conjugated anti-B220 antibody and analyzed on a FACSCalibur flow cytometer Only CD45$^+$ cells were evaluated Differences between the groups were statistically significant ($P < .05$) for all tested cell populations (C) DCs were generated with GM-CSF and IL-4 from HPCs as described. Irradiated at 150 Gy. and cultured with lymph node cells isolated from control allogeneic BALB/c mice at different ratios Cell proliferation was measured in triplicates as described in "Materials and methods." Values are the average ± SE from 2 performed experiments. Differences between the groups were statistically significant ($P < .05$) at all LN cell/DC ratios (D) HPCs were cultured on control or Jagged-1 fibroblasts with FL and IL-4 for 7 days Cells were analyzed as described in Figure 6B Differences between the groups were statistically significant ($P < .05$) for all tested cell populations with the exception of CD11c$^+$B7-2$^+$ cells (E) Bone marrow–enriched HPCs were cultured with GM-CSF or GM-CSF and IL-4 on control and Jagged-1 fibroblasts On day 5. CD45$^+$ cells were isolated and nuclear protein was used for CBF-1–binding activity as described in Figure 3 Lane 1. 50-fold excess of unlabeled "cold" probe; lane 2. mutant probe; lane 3. HPCs cultured with GM-CSF on 3T3-MSCV; lane 4. cells cultured with GM-CSF on 3T3-Jagged-1 fibroblasts; lane 5. HPCs cultures with GM-CSF and IL-4 on 3T3-MSCV fibroblasts; lane 6. cells cultured with GM-CSF nd IL-4 on 3T3-Jagged-1 fibroblasts

(CpG) was used instead of LPS It also did not change the effect of Notch-1 on DC differentiation

Recently, it has been reported that a Jagged-1–soluble peptide can mature human monocyte-derived DCs [18] We asked whether cell-bound Jagged-1 could have the same effect. Immature DCs were generated from HPCs with GM-CSF and IL-4 for 5 days without the presence of fibroblasts. On day 5, cells were transferred onto Jagged-1 or control fibroblasts and cultured for 48 hours prior to DC phenotype analysis No differences were found between these 2 groups of cells in the expression of CD11c, CD11b, MHC class II, B7-2, or CD40 The ability of DCs to stimulate allogeneic T cells also remained the same (data not shown). Addition of

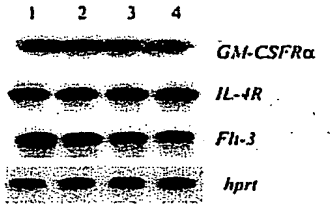

Figure 8. Jagged-1 ligation had no effect on the expression of *GM-CSFRα*, *IL-4R*, or *Flt-3* in HPCs. Bone marrow–enriched HPCs were cultured with GM-CSF on control or Jagged-1 fibroblasts. After 48 hours or 72 hours of incubation CD45$^+$ cells were isolated; RNA was extracted and used in reverse transcription-PCR followed by Southern blotting as described in "Materials and methods." Lanes 1 and 2, HPCs cultured on 3T3-MSCV (1) or on 3T3-Jagged-1 (2) fibroblasts for 48 hours; lanes 3 and 4, HPCs cultured on 3T3-MSCV (3) or on 3T3-Jagged-1 (4) fibroblasts for 72 hours.

TNF-α or LPS resulted in activation of DCs, which was manifested in up-regulation of MHC class II, B7-2, and CD40. However, no differences between cells incubated on control or Jagged-1 fibroblasts were found. These data indicated that under these experimental conditions cell-bound Jagged-1 did not activate DCs.

Discussion

The data presented in this report indicate that Notch signaling is necessary but not sufficient for the differentiation of DCs. Lack of Notch-1 in ES cells significantly impaired their differentiation into DCs. We have previously found that Notch is required to maintain normal levels of nuclear factor κB (NF-κB) subunits in HPCs.[19] It has been reported that RelB, one of the subunits of NF-κB, is critically important for differentiation of one particular type of DCs—myeloid-related DCs.[27] Several subsets of DCs in mice have been described: myeloid-related, lymphoid-related, and plasmacytoid cells. However, specific markers for lymphoid and myeloid DCs are not well defined. CD8α, previously used as a marker of lymphoid DCs, was later found to be expressed on both committed lymphoid and myeloid progenitors. Another approach to separate these 2 populations is staining with anti-CD11c and anti-CD11b antibodies. CD11c$^+$CD11b$^+$ cells were characterized as myeloid-related DCs and CD11c$^+$CD11b$^-$ cells as lymphoid-related DCs (for a review, see Shortman and Liu[4]). Although these criteria are not absolute, they nevertheless allowed for more precise characterization of DC populations. We asked whether Notch signaling might be specifically important for only one subset of DCs. To address this question we generated all subtypes of DCs from HPCs using FL. Highly enriched HPCs obtained by negative selection of lineage-specific cells have been used. Previous studies have demonstrated that these cells are adequate for the analysis of DC differentiation in vitro. HPCs isolated from Notch-1-AS-Tg mice produced the same level of myeloid-related (CD11c$^+$CD11b$^+$), lymphoid-related (CD11c$^+$CD11b$^-$), or plasmacytoid (CD11c$^+$ B220$^+$Gr-1$^+$) DCs as HPCs isolated from control mice, which was consistent with previous observations in Notch-1 conditionally knockout mice. However, when we analyzed the phenotype and function of DCs within each population we found that DCs generated from Notch-1–deficient HPCs had significantly lower levels of MHC class II and costimulatory molecules and a lower ability to stimulate allogeneic MLRs than control HPCs. These data confirmed the observations made in ES cell system and clearly indicated that Notch-1 is critically important for the differentiation of DCs. The differences in the magnitude of the effect between Notch-1 knockout ES cells and Notch-1–deficient HPCs could be explained by the fact that HPCs from Notch-1-AS-Tg mice still have about half of the control level of Notch-1 protein,[19] whereas Notch-1 knockout ES cells were completely devoid of Notch-1.

Using bone marrow chimeric mice with conditionally knockout Notch-1, Radtke and colleagues found that neither thymic nor peripheral DCs were affected by Notch-1 deficiency.[11] However, direct evaluation of DC phenotype and function in mice is very difficult, because of the very low proportion of these cells in tissues. Therefore, DC fractions in that study were enriched using gradient centrifugation.[11] However, although this procedure is adequate for obtaining enriched DCs, it is much less suitable for the evaluation of the total populations of DCs due to considerable loss of cells. In addition, no expression of costimulatory molecules was analyzed nor were functional studies with isolated DCs performed. As we demonstrated here, cells generated from Notch-1–deficient HPCs may have normal expression of CD11c, but significantly decreased B7-2 expression and functional activity. Probably the most accurate evaluation of DC populations in these mice can be achieved after in vivo stimulation with FL and direct assessment of DC phenotype and function.

Is Notch signaling sufficient to promote DC differentiation? We activated Notch in HPCs using a cell-bound ligand, which most closely reflects actual interaction between BMS cells and HPCs in the bone marrow. Notch ligand Jagged-1 was selected because it is expressed on BMS.[28] Among members of Notch family, Notch-1 and Notch-2 are expressed in multiple lineages of hematopoietic cells. Notch-1 in particular appears to be regulated during myeloid differentiation.[29]

In the presence of all tested growth factors activation of Notch resulted in decreased production of mature DCs or macrophages. This was associated with accumulation of ImCs with Gr-1$^+$CD11b$^+$ phenotype. These data were consistent with the results obtained previously on 32D myeloid cells[14] and very recently on HPCs with activation of Notch via Delta-1.[30] ImCs were enriched for HPCs and differentiated into mature DCs or macrophages once Jagged-1–expressing stroma was removed. It indicates that uninterrupted Notch signaling is required to keep cells in relatively undifferentiated state. Our data demonstrate that Notch activation in HPCs does not affect the expression of the receptors for main growth factors and cytokines involved in DC differentiation: GM-CSF, FL, or IL-4. It suggests that Notch effects are not mediated by increased sensitivity of HPCs to these cytokines. At this time we cannot exclude a possibility that expression of Jagged-1 on fibroblasts may affect expression of other molecules able to influence DC differentiation. This will require further testing.

IL-4 induced differentiation of DCs in the presence of Notch signaling. This phenomenon can be explained by the fact that activation of Notch leads to accumulation of the DC precursors. IL-4 apparently is able to override Notch-induced inhibition of DC differentiation, which eventually results in accumulation of mature DCs. Our data indicate that IL-4 does not interfere with Notch activation and apparently affects DC differentiation via other mechanisms currently under investigation.

Activation of Notch via cell-bound Jagged-1 ligand did not activate already differentiated DCs. This was in apparent contrast to recent data from Weijzen and coauthors, who showed that a Jagged-1–derived peptide was able to induce DC activation.[18] It is possible that the level or duration of Notch activation is different between soluble and cell-bound ligands. If the soluble peptide is metabolized in culture, it would result in a transient Notch signal, which is then extinguished, whereas ligand expressed on the

surface of feeder cells generates a continuous signal In this case, exposure to Jagged peptide may mimic Notch stimulation followed by withdrawal of the signal, and trigger maturation

Thus, our data suggest a model of DC differentiation involving Notch In bone marrow, differentiation of DCs is controlled by cytokines and by direct cell-cell contact of HPCs with BMS cells Activation of Notch signaling results in the accumulation of precursors of DCs and macrophages but prevents their terminal differentiation As soon as the influence of BMS is terminated (when precursors leave bone marrow) those cells undergo rapid differentiation into macrophages or DCs Such spatial control of cell differentiation may be necessary for keeping functionally competent mature DCs away from the bone marrow, thus maintaining homeostasis Bacterial products such as LPS or CpG were not able to promote final steps of DC differentiation in the presence of Notch signaling Apparently, their role is limited to activation of DCs on periphery Our data also indicate that strong selection signals such as IL-4 may override that spatial control

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Functional antigen-presenting leucocytes derived from human embryonic stem cells in vitro

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Summary

Background Differentiated cells derived from pluripotent human embryonic stem (hES) cells offer the opportunity for new transplantation therapies. However, hES cells and their differentiated progeny express highly polymorphic MHC molecules that serve as major graft rejection antigens to the immune system of allogeneic hosts. To achieve sustained engraftment of donor cells, strategies must be developed to overcome graft rejection without broadly suppressing host immunity. One approach entails induction of donor-specific immune tolerance by establishing chimeric engraftment in hosts with haemopoietic cells derived from an existing hES cell line. We aimed to develop methods to efficiently differentiate hES cells to haemopoietic cells, including immune-modulating leucocytes, a prerequisite of the tolerance induction strategies applying to hES cell-mediated transplantation.

Methods We developed a method to generate a broad range of haemopoietic cells from hES-generated embryonic bodies in the absence of murine stromal feeder cells. Embryonic bodies were further cultured in the presence of haemopoietic cytokines. In addition to flow cytometric analyses of haemopoietic cell markers, we analysed the hES cell-derived haemopoietic cells by colony-forming assays (for erythroid and myeloid progenitor cells), cytochemical staining, and mixed leucocyte reactions to determine the functional capacity of the generated antigen-presenting cells.

Findings 12 independent experiments were done. When selected growth factors were added, leucocytes expressing CD45 were generated and released into culture media for 6–7 weeks. Under the condition used, both erythroid and myeloid progenitor cells were generated. About 25% of the generated leucocytes acquired MHC class II and costimulatory molecule expression. These hES-derived, MHC class II+ leucocytes resembled dendritic cells and macrophages, and they functioned as antigen-presenting cells capable of eliciting allogeneic CD4 and CD8 T-cell responses in culture.

Interpretation The hES cell-derived antigen-presenting cells could be used to regulate alloreactive T cells and induce immune tolerance for improvement of the transplant acceptance of hES-cell derivatives.

Introduction

Human embryonic stem (hES) cells are pluripotent diploid cells that can proliferate in culture indefinitely [1,2] From these cells we might be able to develop new transplantation therapies to replace diseased or aged cells or tissues [1–5] To this end, researchers need to develop methods with which they can derive from hES cells their required cell types, such as cardiomyocytes or haemopoietic cells, and overcome immune-mediated rejection when these cells are transplanted into hosts that are genetically different (allogeneic) from the hES cell line in use. The HLA system has a central role in the initiation and development of immune rejection. The most important genes are *HLA-A*, *HLA-B*, and *HLA-C* (collectively called class I) and *HLA-DR* and *HLA-DQ* (class II) [6] Class I genes are expressed in virtually all somatic cells whereas expression of class II genes is restricted largely to cells of the immune system, such as dendritic cells, macrophages, and other antigen-presenting cells [7]

MHC molecules are essential in antigen-specific immune activation or tolerance induction because they bind antigenic peptides and present them to a specific T-cell receptor complex. MHC class I molecules preferentially present antigens to CD8+ cytotoxic T cells, whereas CD4+ helper T cells preferentially recognise peptides presented by MHC class II molecules [7] The peptides presented by MHC complex molecules can come from an external source—eg, viral proteins—but mostly they are derived from endogenous proteins encoded by either the nuclear or mitochondrial genome. MHC-restricted antigen presentation by antigen-presenting cells provides the first signal needed to stimulate a specific clone of naive T cells and determines response specificity. However, the ultimate outcome of immune response—either sustained activation (immunogenicity) or tolerance induction—is dictated by other signals from complex interactions between antigen-presenting cells and T cells of various developmental or physiological types. During normal maturation of the haemopoietic and immune systems, tolerance develops to self proteins or antigens. Highly polymorphic MHC molecules (in unmatched allogeneic donor cells) are themselves major foreign antigens presented either directly by donor or indirectly by host antigen-presenting cells, and alloreactive T cells confronting non-identical HLA molecules proliferate vigorously leading to donor cell rejection. Therefore, the

matching of MHC molecules (HLA-A, B, C, DR, and DQ) is necessary to minimise immune rejection However, polymorphisms in many other non-HLA endogenous proteins still provide sources of minor histocompatibility antigens, including highly polymorphic mitochondrial and H-Y gene products, resulting in rejection even in HLA-matched individuals [8]

Currently, immunosuppressive drugs such as ciclosporin are administered to transplant recipients to prevent acute and chronic immune-mediated rejection of allogeneic bone marrow and organ transplants even with best possible MHC matching These drugs, which inhibit all types of T cells non-specifically, result in many side-effects, particularly after long-term use. Fortunately, new developments with haemopoietic chimerism to induce immune tolerance have helped us to greatly reduce or avoid allogeneic rejection responses [8,9] These new strategies include use of haemopoietic stem cells that could reset or reconstitute the haemopoietic and immune systems in myeloablated or non-myeloablated hosts, use of immature dendritic cells or other types of antigen-presenting cells to induce tolerance, and use of transduced dendritic cells with specific genes that will specifically kill or inactivate alloreactive T cells [7–9] The unique properties of ES cells (unlimited growth in culture and pluripotency) allow exploration of new strategies to engineer ES cell-derived donor tissues matching those of the recipients. Several strategies that potentially improve hES cell transplant acceptance have been proposed [10,11] but they all have their advantages and disadvantages. First, we could generate parthenogenetic hES cells if they can be produced from oocytes, but this strategy is restricted to premenopausal females. Second, creation of a patient-specific hES cell by individual somatic cell nuclear transfer into enucleated oocytes (therapeutic cloning) is possible but the presence of the mitochondrial genome in oocytes can contribute minor polymorphic rejection antigens, and the current protocol is far from efficient [12] Third, to achieve best possible MHC matching we could establish large banks of HLA-defined and highly diversified hES cell lines, but this strategy might not be sufficient since minor rejection antigens, are still present and difficult to define. Finally, we could establish immune tolerance (after maximum MHC matching between the patient and an ES cell line in hES cell banks) by preinjection or coinjection of haemopoietic cells derived from the donor ES cells This approach is based on the idea that we can induce immune tolerance to donor-specific antigens by donor haemopoietic cells and promising results in combined allogeneic haemopoietic cell and organ transplantations [7–9]

We aimed to develop methods to efficiently differentiate hES cells to haemopoietic cells, including immune-modulating leucocytes, a prerequisite of the tolerance induction strategies applying to hES cell-mediated transplantation

Methods

Cell culture and differentiation

We cultured the H1 hES cell line (passage 22; WiCell Research Institute, Wisconsin, MI, USA) on primary mouse embryonic fibroblasts that we used as feeder cells We used karyotypically normal hES cells (passage 30–80). The procedure for karyotyping and culturing H1 hES cells on primary mouse embryonic fibroblasts or human feeder cells has been previously described [13] In brief, the hES cell culture medium consists of 80% (v/v) knockout DMEM (Dulbecco's modified eagle medium), 20% (v/v) of the knockout serum replacement, 2 mmol/L L-glutamine, 0·1 mmol/L non-essential aminoacids (all from Invitrogen, Carlsbad, CA, USA), 0·1 mmol/L β mercaptoethanol (Sigma, St Louis, MI, USA), and 4 μg/L basic fibroblast growth factor (Peprotech, Rocky Hill, NJ, USA)

Before differentiation, we passaged hES cells at a high density (1/1 to 1/3 split ratios) onto Matrigel (Becton Dickson Labware, Bedford, MA, USA), as previously described [14] After reaching the full size or confluency, hES cell colonies were incubated with dispase (0·2 g/L, Invitrogen) at 37°C for 45–60 min. Under this condition, we lifted ES colonies intact after digestion and separated them away from the residual feeder cells We resuspended the harvested hES cell colonies in the hES cell medium in the absence of basic fibroblast growth factor; we also added fetal bovine serum (20% final; StemCell Technologies, Vancouver, Canada) To prevent cell attachment to plastic and to induce formation of embryonic bodies, we cultured ES colonies in ultralow-attachment plates (Corning Costar, Cambridge, MA, USA) About 0·1–0·5 million ES cells were incubated in every well of six-well plates and formed 20–30 embryonic bodies Cystic embryonic bodies emerged after 5–20 days in suspension cells When harvested at day 10–20, about 50–80% embryonic bodies were cystic (on average every embryonic body contained about 10 000 cells)

To generate a broad range of haemopoietic cells, including dendritic cells and other antigen-presenting cells, we adapted a protocol developed previously for mouse ES cells [15] We transferred whole embryonic bodies formed in suspension onto tissue culture plates (without dispersion) and allowed them to differentiate into haemopoietic and other cell types in fetal bovine serum-containing media. This medium contained 80% knockout DMEM, 2 mmol/L glutamine, 0·1 mmol/L β mercaptoethanol, 0·1 mmol/L non-essential aminoacids, and 20% fetal bovine serum Furthermore, to stimulate production of haemopoietic progenitor cells and dendritic cells, we added: stem cell factor (100 μg/L), FLT3 ligand (50 μg/L), and thrombopoietin (20 μg/L), which are cytokines widely used to maintain human postnatal haemopoietic stem cells and to expand committed progenitor cells;[16] interleukin-3 (20 μg/L); granulocyte-macrophage

colony-stimulating factor (100 μg/L); and, to enhance possible maturation of lymphoid cells and dendritic cells, we added interleukin 4 (20 μg/L).[7] We purchased all cytokines from Peprotech

Flow cytometric analysis

We harvested undifferentiated adherent hES cells and adherent cells in haemopoietic cell cultures with non-enzymatic cell dissociation solution (Invitrogen). Cells in embryonic bodies were digested with 0·4 U/mL collagenase B (Roche Molecular Biochemicals, Indianapolis, IN, USA) at 37°C for 2 h. We stained harvested suspension or adherent cells with antigen-specific monoclonal antibodies or their isotype controls (mouse IgG1 or IgG2a). The following R-phycoerythrin-conjugated monoclonal antibodies were used for flow cytometric analysis: antibodies against HLA-DR, CD3, CD5, CD14, CD19, CD33, CD83, CD80, and CD86 (Becton Dickinson PharMingen, San Diego, CA, USA); glycophorin A (Immunotech); and CD2, CD7, CD22, CD31, CD34, and CD45 (Caltag Laboratories, Burlingame, CA, USA). In two-colour (antigen) analyses, we used fluorescein-isothiocyanate (FITC)-conjugated CD45 or its isotype (mouse IgG1) control antibody (Caltag) in conjunction with a phycoerythrin-conjugated antibody.

We did fluorescence-activated cell sorter (FACS) analysis with a FACScan or FACSCalibur analyser (Becton Dickinson). We set up the machine such that 10 000 events (cells) were counted and analysed as a test population. The fluorescence intensity (reflecting antigen density on cell surface) from the binding of a specific antibody (conjugated with a fluorochrome) is recorded for each of 10 000 cells in four orders of magnitude. In histograms, cell numbers are plotted as a function of variable fluorescence intensities. The percentage of antigen-expressing cells with specific fluorescence signals above background (≤1% of 10 000 collected events) is automatically calculated by the FACS machine with BD CellQuest Pro software (Becton Dickinson). In dot plots (fluorescence intensities of a given antigen *vs* that of the second antigen or side scatter), every dot represents one of 10 000 cells (or in proportion) analysed automatically by the machine. Since the denominator is 10 000 events, the SE is small

Cell assays

For haemopoietic colony-forming assays, single cells from either non-adherent (suspension) or adherent cell fractions were plated in methylcellulose media (MarrowGro, Quality Biological, Gaithersburg, MD, USA), as previously described.[16] We plated up to 5×10^4 cells in every 35-mm plate in duplicate. After 14 days of culture, we counted colonies resembling either erythroid burst colony-forming units or granulocyte-monocyte colony-forming units. We assessed morphological differentiation of colony-derived progeny on cytospin slides by standard Wright-Giemsa staining or by a modified version with Diff-quick staining kit (Fisher Scientific Company, Swanee, GA, USA). We viewed the cells under a ×100 oil immersion lens. Similarly, to identify different cell types we stained cells harvested

Marker (other names)	Major presence on postnatal human cells	Undifferentiated hES cells*	Cells within embryonic bodies†	Haemopoietic progeny‡
CD2	T and natural killer cells, certain blood myeloid cells	–	N/D	+ (27–37%)
CD3	T cells	–	N/D	–
CD14	Monocytes and macrophages	–	N/D	+ (up to 20%)
CD16	Neutrophils; natural killer cells	–	N/D	+ (up to 15%)
CD19	B cells	–	N/D	–
CD31 (PE-CAM)	Haemopoietic and endothelial cells	–	+	+ (up to 10%)
CD33	Myeloid cells	–	N/D	+ (up to 69%)
CD34	Stem/progenitor cells; endothelial cells	–	+ (5–10%)	+ (up to 3%)
CD40	Antigen-presenting cells such as B cells, dendritic cells, and macrophages	–	N/D	+ (~15%)
CD45 (LCA)	Pan leucocytes	–	–	+ (>90%)
CD56 (N-CAM)	Natural killer cells; neutrophils; neural cells	–	N/D	–
CD80 (B7 1, costimulatory molecule)	Antigen-presenting cells such as dendritic cells, B cells, and macrophages	–	N/D	+ (~16%)
CD83	Mature dendritic cells	–	N/D	+ (8%)
CD86 (B7 2, costimulatory molecule)	Antigen-presenting cells such as dendritic cells, B cells, and macrophages	–	N/D	+ (~30%)
CD90 (Thy-1)	Stem/progenitor cells; thymocytes; non-haemopoietic cells	+	N/D	N/D
CD105 (endoglin)	Haemopoietic and endothelial cells; fibroblasts	–	N/D	N/D
CD117 (C-KIT/SCFR)	Stem/progenitor cells; mast cells	+	+	N/D
CD133 (AC133)	Stem/progenitor cells	+	N/D	N/D
VEGFR2 (FLK1, KDR)	Stem/progenitor cells; endothelial cells	+	N/D	N/D
MHC I (HLA-ABC)	All nucleated cells	+	+	+ (100%)
MHC II (HLA-DR)	Antigen-presenting cells	–	–	+ (~25%)

N/D=not determined. *FACS analysis of undifferentiated (SSEA4+) hES cells. –=<2% cells display signals above background. Non-enzymatic or trypsin/EDTA mediated cell dissociation methods gave similar results. †FACS analysis or section staining when cystic embryonic bodies were first seen. ‡FACS analysis of non-adherent cells harvested at various timepoints from our haemopoietic cultures.

Table: Expression of cell surface markers on undifferentiated hES cells and their haemopoietic progeny

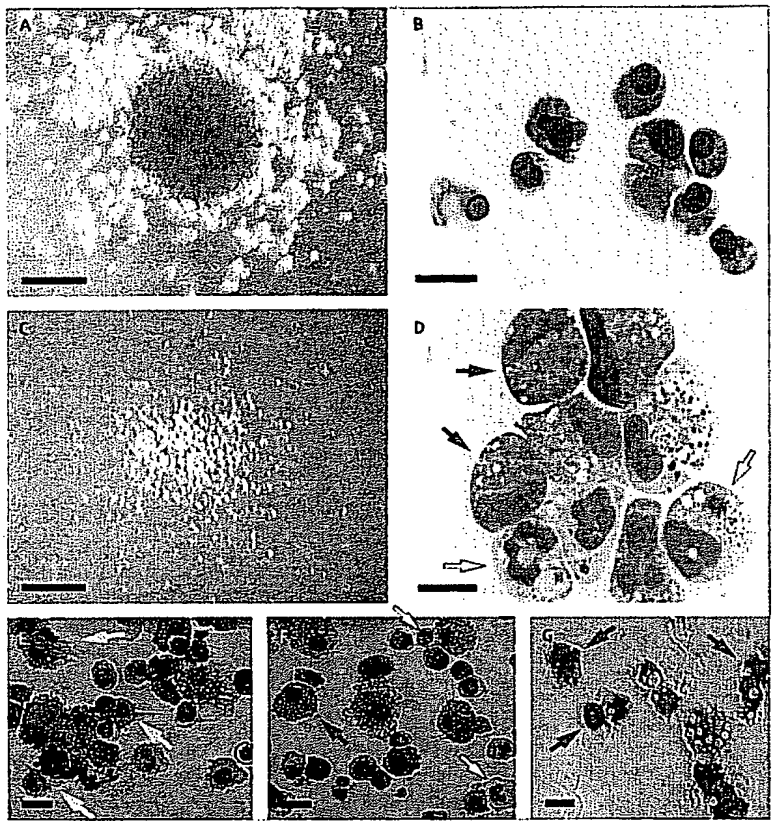

Figure 1: Photomicrographs of colony-forming progenitor cells and other hES-derived haemopoietic cells (A) Erythroid burst colony-forming units (B) Diff-quick stained individual erythroid burst colony-forming units containing nucleated, polychromophilic, or orthochromophilic erythroblast cells typical of those seen in cells derived from bone marrow [11] (C) Myeloid colony (D) Diff-quick stained myeloid colonies containing monocytes (red arrows) and granulocytes (white arrows) (E and F) Wright-Giemsa staining of non-adherent haemopoietic cells Green arrows indicate cells resembling dendritic cells, red arrows point to macrophage-like cells, and white arrows show cells resembling granulocytes (G) α naphthyl acetate esterase staining for macrophages Positive staining for this enzyme is seen in the cytoplasm containing vacuoles Most cells did not stain positively Scale bars: 200 μm in A and B; 20 μm in C–G

from suspension cultures by Wright-Giemsa staining on cytospin slides. The presence of macrophages was further confirmed by their expression of α naphthyl acetate esterase with a staining kit (procedure 91; Sigma)

Differentiated haemopoietic cells obtained from ES cell derivatives in suspension were further cultured for 4 days with granulocyte-macrophage colony-stimulating factor (150 μg/L) and interleukin 4 (20 μg/L) in RPMI-1640 medium with 10% fetal bovine serum Harvested cells were briefly activated by tumour necrosis factor α (20 μg/L) and prostaglandin E2 (10 mg/L) for 4 h, and they were irradiated at 30 Gy to block cell proliferation. We used these cells as stimulators in mixed leucocyte reaction assays to measure antigen-presenting cell-dependent allogeneic T-cell proliferation, as previously described [11] Briefly, peripheral blood mononuclear cells from healthy adult donors were seeded (2×10^5 cells per well, constant) in 96-well plates as responders, with serially diluted hES cell progeny as stimulators (stimulator/responder ratios were 1/8, 1/16, and 1/32) in replicates (n=3–5) in every experiment with mononuclear cells from different donors. As control antigen-presenting cells we used human cord-blood leucocytes, undifferentiated hES cells, and embryonic body-derived hES cells before haemopoietic differentiation as stimulators after irradiation. In selected experiments, purified human CD4+ or CD8+ T cells (10^5 per well) were used to replace mononuclear cells as responders (n=3). With the MACS CD4 or CD8 T-cell purification systems (Miltenyi Biotec, Auburn, CA, USA) more than 99% of purified cells expressed CD4 or CD8 (from about 45% and 15%, respectively, in total mononuclear cells) After 3 days of coculture, we pulsed cells with 1 μCi per well of ^{3}H-thymidine and harvested them 18–20 h later with a Packard Micromate cell harvester (Packard BioScience, Meriden, CT, USA). We measured ^{3}H-thymidine incorporation (counts per minute, cpm) with a Packard Matrix 96 direct β counter, as previously described [11]

Statistical analysis

We used Microsoft Excel (version X for Macintosh) software for data management (calculation of mean and SD), scatter plots, and Student's *t* tests (two-sided, unequal variance) When the number of replicates (n) was small (n=3–5) in every group in individual experiments (not pooled data from several similar experiments), we used the non-parametric Mann-Whitney test (also known as Wilcoxon two-sample test). This test was run unsupervised with the SAS 8 2 package (SAS Institute, Cary, NC, USA). We judged findings to be significant if p (null hypothesis) was 0 05 or less by both tests

Role of the funding source

The sponsor had no role in study design, data collection, data analysis, data interpretation, writing of the report, or in the decision to submit for publication

Results

The table summarises the expression of 21 surface markers on undifferentiated hES cells. Some commonly used markers associated with haemopoietic progenitor cells and committed lineages were also expressed in undifferentiated hES cells, including Thy-1/CD90 and MHC class I However, CD45 (leucocyte common antigen), CD34, and MHC class II (such as HLA-DR) were not expressed in undifferentiated hES cells defined as SSEA4+ cells. We therefore chose acquired expression of these markers (such as CD45 and MHC class II) to monitor differentiation towards haemopoietic lineages

Embryonic bodies attached firmly to culture plates within 2 days. During the next 2 weeks, many types of adherent cells proliferated rapidly then migrated out and formed adherent cells surrounding embryonic bodies. After 5 days, haemopoietic-like cell clusters emerged on the edge of the attached embryonic body cell masses, apparently in tight association with the newly formed adherent cells These clusters were similar to those seen in the mouse ES cell system and to the early events of haemopoietic differentiation from rhesus monkey ES cells [15-17] Most of these haemopoietic cell-like clusters disappeared from adherent cell layers in the following 5 weeks after they reached a diameter of 0·5 mm Eventually, these adherent blastic cells disappeared and most probably moved into suspension in a manner typical of fetal and postnatal haemopoietic cells

Within 7–10 days after embryonic bodies were plated, haemopoietic-like cells started to appear in suspension Most non-adherent cells appeared as single cells, but small cell clusters with dendritic appearance were also visible. These non-adherent cells were collected weekly (week 2–6); mean total number of harvested cells was 2·32 million per well (SD 0 80; n=4), containing about 40 embryonic bodies Starting at week 2 when sufficient numbers of cells were available, both non-adherent and adherent cell fractions were analysed by colony-forming assays (figure 1) and FACS analysis for haemopoietic marker expression (figure 2) Observed erythroid colonies resembled erythroid burst colony-forming units; granulocyte-monocyte colony-forming units were also detected in both non-adherent and adherent cell fractions (figure 1) At all timepoints, more granulocyte-monocyte colony-forming units (4–5 fold) were seen than erythroid burst colony-forming units (data not shown). In one representative experiment, frequencies of total colony-forming cells (mean [SD], n=2) measured every week were: 35 5 (1 5), 18·5 (1 5), 14 (2), 32 (2), and 10 (1) per 10^5 non-adherent cells; and 77 (2), 49 (1), 18 (0), 15 (1), and 2 (0) per 10^5 adherent cells; for weeks 2–6, respectively.

Figures 2 and 3 show FACS analyses of non-adherent and adherent cells harvested at week 2. Nearly all

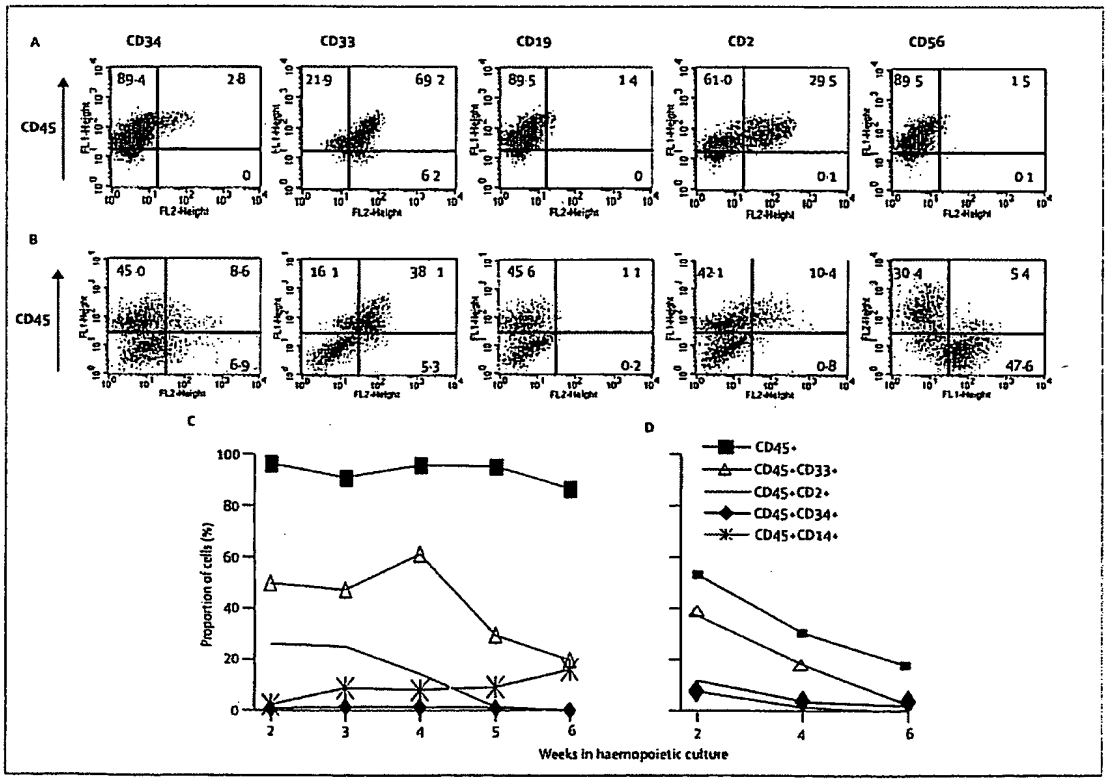

Figure 2: **FACS analysis of multiple types of hES-derived haemopoietic cells**
Non-adherent (A) or adherent cells (B) harvested at week 2 Committed haemopoietic cells were identified by CD45 (*y* axis) together with other lineage markers (*x* axis), indicated at the top of every panel. Background (≤1%) was identified by staining with isotype-matched control antibodies. Percentages of positively stained cells are based on 10 000 cells counted automatically by the machine. Percentages of cells in (C) non-adherent and (D) adherent cell fractions at week 2 and later timepoints

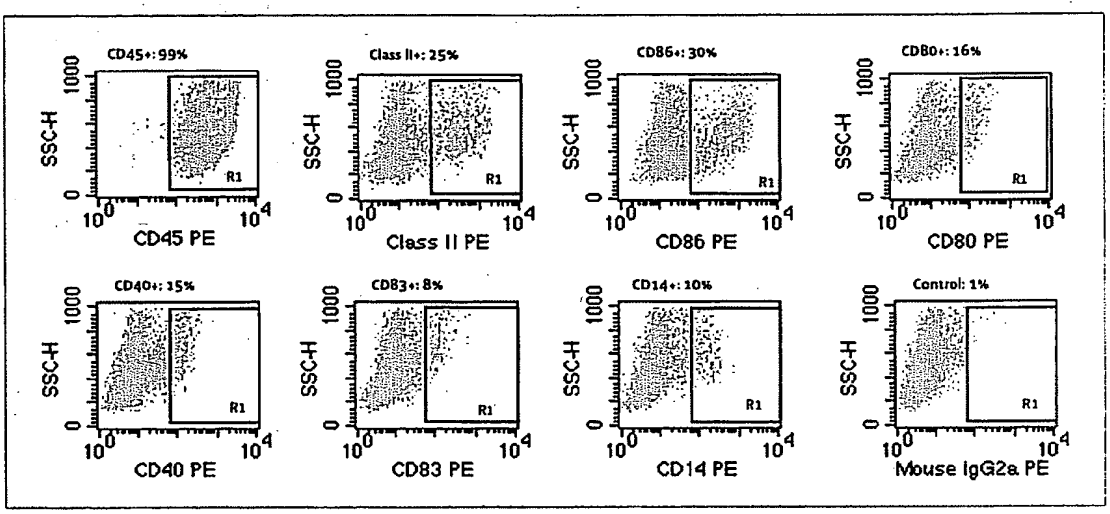

Figure 3: **FACS analysis of ES-derived leucocytes after stimulation**
Side scatter (SSC-H; *y* axis) was plotted against expression of various leucocyte markers (*x* axis). The positive signal above background (shown in the lower right corner) was gated (R1) accordingly. Percentage of positively stained cells (in R1, out of 10 000 cells counted) is denoted at the top of every panel

non-adherent cells were CD45+ (96%), confirming their haemopoietic nature. Notably, 53 6% of adherent cells were also CD45+, indicating that many haemopoietic cells were also present after non-adherent cell harvest. More CD45+CD34+ (haemopoietic stem and progenitor) cells were recorded in the adherent cell fraction (8 6%) than in the suspension (non-adherent cell) fraction (2 8%) at week 2. The percentages of these cells in the non-adherent and adherent cell fractions fell to background levels (≤1%) at weeks 3 and 6, respectively (figure 2). CD34+CD45− cells were also noted in the adherent fraction, which were probably endothelial cells; these cells remained during the analysis period (data not shown).

CD33+CD45+ cells (characteristic of myeloid lineages) were readily detectable in both the non-adherent and adherent cell fractions (figure 2) as were low percentages (about 5%) of CD14+CD45+ cells (data not shown). Under the culture conditions, non-significant numbers of B lymphoid (CD19+) cells were detected in each fraction. Unexpectedly, CD45+ cells coexpressing CD2 (a marker usually seen on T cells and natural killer lymphoid cells) were detected in both fractions (figure 2). The reproducible presence of high percentage (27–37%) CD2+ non-adherent cells in every experiment (n=5) prompted us to further determine whether they could be lymphocytes. However, the isolated non-adherent cells did not express other lymphoid markers such as CD3, CD5, and CD7 (data not shown). Although about 50% of CD2+ cells expressed CD16 (in both natural killer cells and granulocytes) they did not have other markers associated with natural killer cells such as CD56 (figure 2) and CD94 (data not shown). Since CD2+ monocytes and dendritic cells are reported in blood. CD2 is no longer regarded as an exclusive lymphocyte (natural killer and T) cell marker.[19,20] These data are consistent with the notion that CD2+CD16+ cells are probably myeloid in origin.

At later timepoints of haemopoietic differentiation (weeks 3–6), more non-adherent cells than adherent cells expressed CD14 (figure 2) and MHC class II and costimulatory markers such as CD80 or CD86 (see below). The MHC class II complex is selectively expressed in such antigen-presenting cells as dendritic cells, macrophages, and B cells, whereas nearly all nucleated cells express MHC class I. Since we did not detect (CD19+) B cells, we directly analysed the presence of macrophages and dendritic cells in hES-derived leucocytes found in suspension. Wright-Giemsa staining revealed the presence of many cell types, including morphologically distinct cells resembling dendritic cells, macrophages, and granulocytes (figure 1). The presence of macrophages was further confirmed by cytoplasmic expression of α naphthyl acetate esterase (figure 1).

We further analysed the non-adherent cell population after brief activation of dendritic cells and antigen-presenting cells with tumour necrosis factor α and prostaglandin E2. About 25% of cells expressed moderate to high amounts of MHC class II (HLA-DR) and the costimulatory molecule CD86 (figure 3). Cells expressing CD40 (a marker for antigen-presenting cells), CD83 (a dendritic cell marker), or CD14 (a macrophage and monocyte marker) with a high side scatter were also detected (figure 3), confirming the findings on Wright-Giemsa staining that dendritic cells

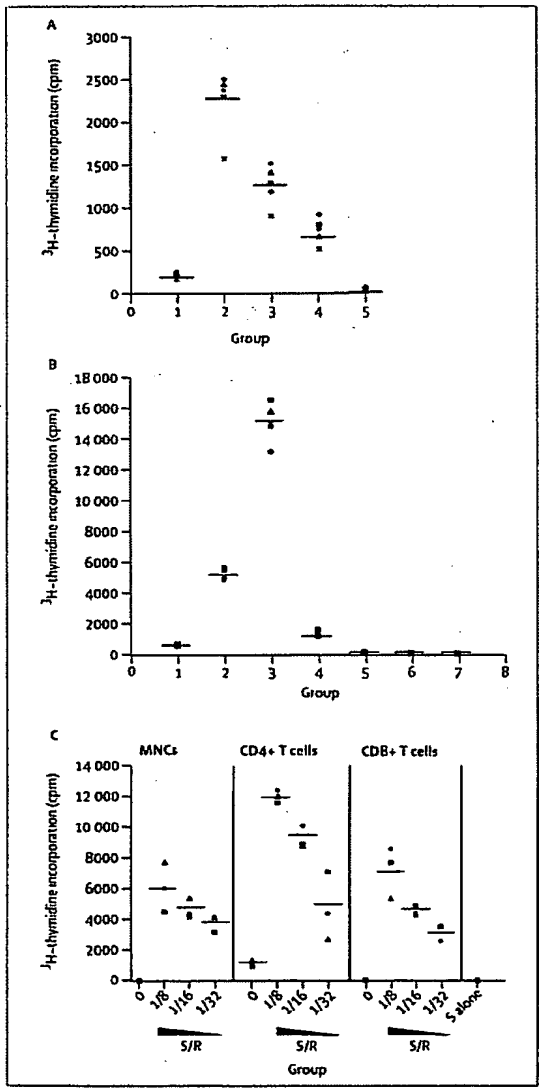

Figure 4: **Mixed leucocyte reaction assays**
(A) 2×10^5 human blood leucocytes (responders [R], n=5) seeded without (1) or with (2–4) hES cell-derived haemopoietic cells used as antigen-presenting cells (stimulators [S]) to stimulate T-cell proliferation. S/R ratios (2–4) were 1/8, 1/16, and 1/32, respectively. (5) Maximum number of S (25 000 cells, irradiated and mitotically inactive) seeded alone as a negative control. Horizontal bar=mean value. For all comparisons, p=0·0122, by Mann-Whitney test. (B) Blood leucocytes (R, n=5) seeded alone (1) or with irradiated antigen-presenting cells (S; S/R=1/8) in groups (2–4). (2) hES-derived haemopoietic cells, as above; (3) allogeneic blood leucocytes from cord blood; (4) non-haemopoietic progeny of hES cells. (5–7) Corresponding unseeded cells in (2–4). For all comparisons, p=0·0122, by Mann-Whitney test. (C) Purified CD4+ or CD8+ T cells (10^5 cells per well, n=3) used as responders (R), in comparison with total mononuclear cells (MNCs, 2×10^5 cells per well). Stimulators (S) are hES cell-derived haemopoietic cells as antigen-presenting cells with different responders (R). S/R ratios: 0 (R alone), 1/8, 1/16, and 1/32. S alone: irradiated antigen-presenting cells (maximum dose, 25 000 cells).

and macrophages were present (figure 1). However, most dendritic cells and macrophages seemed immature or not fully activated (figure 3).[7]

To test whether these hES cell-derived haemopoietic cells consisting of MHC class II+ cells could indeed function as antigen-presenting cells, we did mixed leucocyte reaction assays to measure (allogeneic) T lymphocyte reaction. The non-adherent haemopoietic derivatives of hES cells were briefly activated, irradiated, and used as antigen-presenting cells (stimulators) in mixture with blood leucocytes as a source of responding T cells (figure 4). Dose-dependent proliferation of responding T cells was recorded as ^{3}H-thymidine incorporation. The hES cell-derived haemopoietic cells (about 25% expressing MHC class II) stimulated T-cell proliferation significantly at a dose as low as 1/32 (figure 4); activities of the hES-derived leucocytes harvested at weeks 3–5 were similar (data not shown). However, the antigen-presenting cell activities were always lower (2–5-fold) than those of irradiated postnatal blood leucocytes from three different donors (figure 4).

We also tested antigen-presenting cell activities of undifferentiated hES cells or their progeny after differentiation mediated by embryonic bodies. Differentiated hES cells before the directed haemopoietic differentiation step showed a low but detectable amount of antigen-presenting cell activity (figure 4); undifferentiated cells did not show activity (data not shown). We next did the same assay with purified CD4 or CD8 T cells that require antigen-presenting cells expressing MHC class II or class I antigen complexes, respectively. Figure 4 shows the results of a representative experiment using the same batch of hES-derived haemopoietic derivatives as antigen-presenting cells and 10^5 CD4 T cells, 10^5 CD8 T cells, or 2×10^5 unfractionated mononuclear cells (containing about 45% CD4 and 15% CD8 T cells) as responders. Highly purified CD4 T cells seemed to show a greater stimulation in response to hES-derived antigen-presenting cells than CD8 T cells and T cells in mononuclear cell mixtures (figure 4).

Discussion

We have provided strong evidence that hES cell-derived leucocytes can function as antigen-presenting cells and directly stimulate allogeneic CD4 and CD8 T cells. We also have extended previous analysis of haemopoietic markers expressed on the H1 hES cell line [21–23].

Kaufman and colleagues[21] provided evidence of haemopoietic differentiation from hES cell lines by showing the formation of erythroid and myeloid progenitor cells in culture. Our method generated multiple lines of haemopoietic cells from hES cells in culture without use of exogenously added stromal feeder cells of either animal or human origin. The absence of feeder cells makes easy not only counting and analysis of hES cell derivatives but also study of growth factor

requirements of hES cell-initiated haemopoiesis. The absence of murine stromal cells further reduces the risk of rodent pathogen transmission to hES cell derivatives generated in coculture, especially when they are destined for clinical use. In our culture system, human adherent cells generated from cystic embryonic bodies seemed to be sufficient in the presence of haemopoietic cytokines. This finding accords with those of a study to derive haemopoietic cells after embryonic body formation.[14] The developmental stages of haemopoietic differentiation from hES cells are similar to those shown with mouse ES cells with the embryonic body formation approach [3,15]

Similar to findings of previous studies,[21,24] we noted the formation of erythroid and myeloid progenitor cells in our hES cell differentiation system. Moreover, we also saw the generation of immune-modulating leucocytes such as dendritic cells and macrophages. About 25% of cells in suspension acquired expression of MHC class II and expressed CD80 or CD86 costimulatory molecules. More macrophages than dendritic cells were present in this population, based on both morphological and FACS analyses. Collectively, these hES cell-derived leucocytes functioned as antigen-presenting cells in stimulation of purified allogeneic CD4 and CD8 T cells in mixed leucocyte reaction assays. Our culture system of hES cell-initiated haemopoiesis provides a foundation for future improvements to study early developmental events of human blood and immune cell formation. Our findings also serve as a step to investigate induction of immune tolerance with hES cell-derived haemopoietic cells.

In addition to their uniqueness as a model system to study human cell biology and immunology, differentiated cells derived from pluripotent hES cells offer the opportunity for new transplantation therapies. To achieve sustained engraftment of hES-derived donor cells, strategies must be developed to overcome graft rejection without broadly suppressing host immunity. Creation of a patient-specific ES cell line by undertaking patient's somatic cell nuclear transfer is now feasible[12] but inefficient, maybe insufficient, and associated with ethical concerns. An alternative approach to avoid graft rejection entails induction of donor-specific immune tolerance to cells and tissues from a selected hES cell line. To achieve the best graft acceptance we should select from hES cell banks a cell line, the HLA type of which is the closest possible match to that of the patient. Since multiple cell types could be derived from the same ES cell line, to derive MHC-identical haemopoietic cells (for tolerance induction) and a second (therapeutic) tissue such as pancreatic islet cells or cardiomyocytes is possible.

Haemopoietic cells are fairly easy to engraft after intravenous injection and can induce immune tolerance and form stable chimerism in allogeneic but MHC-matched hosts.[8,9,25] Because multiple cell types derived from one hES cell line are genetically identical (including HLA genes), engraftment of the ES cell-derived haemopoietic cells might lead to induction of immune tolerance and permit better subsequent graft acceptance of the second cell type.[10,11] This approach is not specific to HLA types of individual patients or hES cell lines, and has been used in transplantation of postnatal tissues or organs.[8–11,25] It is also substantiated by findings with rat ES-like cells that MHC class II+ leucocytes were generated in vivo and induced long-term acceptance of cardiac graft (with a haplotype identical to the rat ES-like cells) in a fully MHC-mismatched recipient rat.[26]

However, undifferentiated hES cells do not express MHC class II and could not function as antigen-presenting cells to stimulate T cells. Commitment to haemopoietic differentiation is required to obtain MHC class II expression and raised antigen-presenting cell activities in stimulation of T cells in culture. Because undifferentiated hES cells can form tumour (teratoma) and lack antigen-presenting cell activity, direct injection of hES cells into recipients to induce immune tolerance is not a safe approach. For safe and effective induction, we will probably need to differentiate hES cells towards haemopoietic commitment in vitro before transplantation. In the future, we will need to test our ability to generate transplantable antigen-presenting cells or haemopoietic stem cells (that subsequently generate antigen-presenting cells in vivo after transplantation) from differentiated hES cells.

Contributors

X Zhan and G Dravid contributed equally to this work. X Zhan and I Cheng initiated the project. G Dravid was responsible for cell analysis and antigen-presenting cell functional assays. Z Ye and H Hammond provided support at every stage of this project. M Shamblott contributed analysis of cell types within embryonic bodies. J Gearhart provided insight into many issues of ES cell transplantation. I Cheng conceived and supervised the project, and wrote the report.

Conflict of interest statement

All authors are employees of Johns Hopkins University and declare no conflict of interest.

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Directed differentiation of dendritic cells from mouse embryonic stem cells

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Dendritic cells (DCs) are uniquely capable of presenting antigen to naive T cells, either eliciting immunity [1] or ensuring self-tolerance [2]. This property identifies DCs as potential candidates for enhancing responses to foreign [3] and tumour antigens [4], and as targets for immune intervention in the treatment of autoimmunity and allograft rejection [1]. Realisation of their therapeutic potential would be greatly facilitated by a fuller understanding of the function of DC-specific genes, a goal that has frequently proven elusive because of the paucity of stable lines of DCs that retain their unique properties, and the inherent resistance of primary DCs to genetic modification. Protocols for the genetic manipulation of embryonic stem (ES) cells are, by contrast, well established [5], as is their capacity to differentiate into a wide variety of cell types *in vitro*, including many of hematopoietic origin [6]. Here, we report the establishment, from mouse ES cells, of long-term cultures of immature DCs that share many characteristics with macrophages, but acquire, upon maturation, the allostimulatory capacity and surface phenotype of classical DCs, including expression of CD11c, major histocompatibility complex (MHC) class II and co-stimulatory molecules. This novel source should prove valuable for the generation of primary, untransformed DCs in which candidate genes have been overexpressed or functionally ablated, while providing insights into the earliest stages of DC ontogeny.

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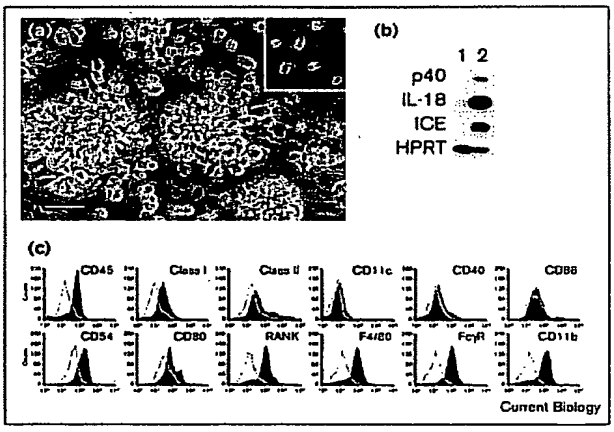
Results and discussion

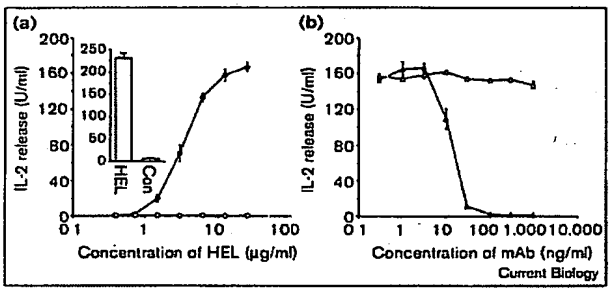
ESF116 is a novel ES cell line derived from the epiblast of a delayed-implanting CBA/Ca blastocyst [7] and is karyotypically male and germ-line competent. This line could be maintained undifferentiated on mouse embryonic fibroblasts but produced embryoid bodies (EBs) when grown in suspension. After culture for 14 days, EBs were plated onto tissue culture plastic in medium supplemented with growth factors implicated in the differentiation of DCs. These conditions promoted the adherence of EBs and the outgrowth of a variety of differentiated cell types. Of all cytokines tested (see Supplementary material), the combination of granulocyte/macrophage colony stimulating factor (GM-CSF) and interleukin (IL)-3 uniquely supported the development of cells with an appearance suggestive of DCs within 4 days of culture. These cells expanded rapidly over the ensuing 7–10 days to form lightly adherent clusters (Figure 1a), highly reminiscent of those observed in cultures of immature DCs derived from bone marrow (bmDCs). Cells released from these clusters seeded uncolonised areas of the dish and are referred to here as 'emigrants'. These cells displayed typical dendritic morphology (Figure 1a, inset) and formed

Figure 1



Characterisation of emigrants derived from ESF116 (a) Phase contrast micrograph showing the development of clusters of cells, reminiscent of cultures of bmDCs. Inset, emigrants released from clusters showing characteristic dendritic morphology, including dendrites and veils of cytoplasm. The scale bar represents 50 μm. (b) RT–PCR analysis of RNA from ESF116 (lane 1) and emigrants derived from them (lane 2), showing upregulation of IL-12 p40, IL-18 and ICE upon differentiation. Methods used for the analysis of RNA by RT–PCR, and details of the primers used, have been described previously [15]. Expression of hypoxanthine-guanine phosphoribosyl transferase (HPRT) was used as a control. (c) Surface phenotype of emigrants. Filled histograms, levels of expression of the markers indicated; open histograms, background staining with irrelevant species- and isotype-matched monoclonal antibodies.

Figure 2



Antigen processing and presentation (a) Processing of HEL by live (filled circles) or fixed (open circles) emigrants measured as a function of IL-2 release by 2G7.1. The inset shows the specificity of the response to HEL by comparison with an irrelevant antigen, conalbumin (con). (b) Inhibition of antigen presentation by emigrants upon addition of the monoclonal antibody 17-3-3S, which is specific for H-2E^k (filled triangles), but not UPC-10, an irrelevant control monoclonal antibody (open triangles). Assays of antigen processing and presentation were performed as described previously [16]

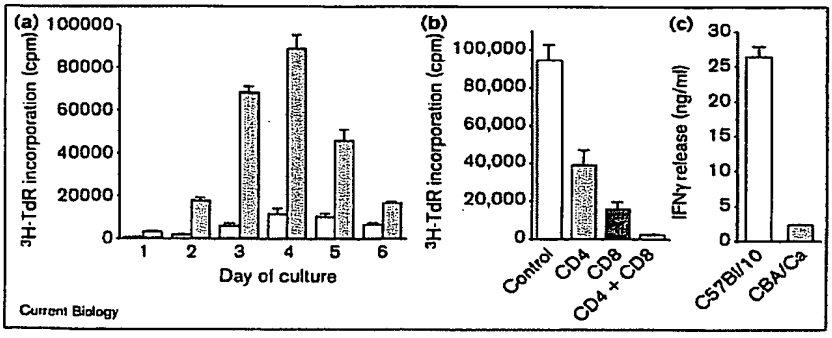
long-term cultures capable of regenerating rapidly upon routine harvesting.

To characterise these cells, we purified RNA from the ES cell line and from highly enriched populations of emigrants derived from them. Although ESF116 consistently proved negative, reverse transcription (RT)–PCR analysis of emigrants revealed the presence of mRNA for the p40 subunit of IL-12, IL-18 and IL-1β converting enzyme (ICE), required for the processing of pro-IL-18 to its bioactive form (Figure 1b). These findings are suggestive of an immunostimulatory function, both cytokines being actively secreted by DCs [8,9] and known to be responsible for biasing responding T cells towards a T helper 1 (Th1) phenotype. Consistent with this possibility, these cells expressed CD45, confirming their hematopoietic origin, together with class I and II MHC determinants (Figure 1c), the latter being found at low-to-intermediate levels on the cell surface because of their retention within intracellular compartments (data not shown). In contrast, these cells were negative for CD11c and the co-stimulatory molecules CD40 and CD86, but consistently expressed moderate levels of CD54, CD80 and RANK. Although they failed to express lineage-specific markers for T cells, B cells and neutrophils (data not shown), this population routinely expressed F4/80, FcγRII/III and CD11b, a phenotype shared by immature bmDCs and macrophages. Significantly, however, replating of these cells in macrophage colony stimulating factor (M-CSF) failed to promote proliferation or changes in their morphology and phenotype, suggesting that they may already have diverged from macrophage precursors. To investigate their possible commitment to the DC lineage, we therefore assessed their propensity for antigen processing and their ability to stimulate primary T-cell responses.

The ability of emigrants to process foreign antigen was measured as a function of their ability to stimulate 2G7.1, a T-cell hybridoma specific for hen egg lysozyme (HEL) in the context of H-2E^k (Figure 2a, inset). Figure 2a shows the dose-dependent release of IL-2 by 2G7.1 cultured in the presence of live cells pulsed with whole HEL, but their failure to respond following prior fixation of emigrants with paraformaldehyde to prevent endocytosis. Furthermore, presentation of HEL was entirely inhibited upon addition of a monoclonal antibody specific for H-2E^k, but not a control monoclonal antibody (Figure 2b), confirming presentation of the antigen in a classic, MHC-restricted fashion.

We next investigated the ability of emigrants to stimulate primary T-cell responses in the mixed leukocyte reaction (MLR). Mitomycin C-treated stimulators were cultured with purified T cells from either syngeneic or allogeneic mice, and proliferative responses measured at 24 hour intervals. Figure 3a shows significant proliferation of naïve

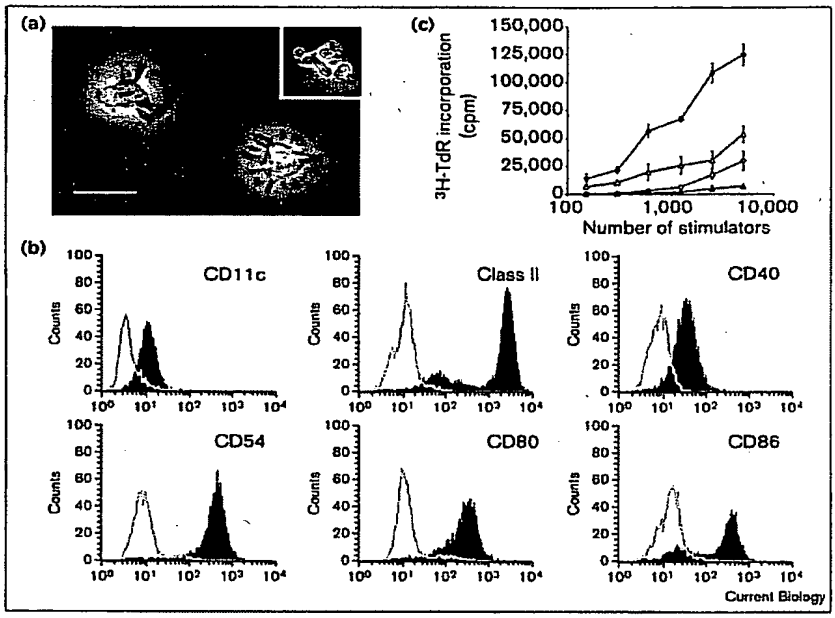
Figure 3



Immunostimulatory capacity of emigrants (a) Kinetics of the proliferative response of naïve allogeneic (C57Bl/10; grey bars) and syngeneic T cells (CBA/Ca; white bars) upon co-culture with emigrants. (b) Inhibition of the proliferative response on day 4 of culture by blocking monoclonal antibody to CD4 (YTS177) and/or CD8 (YTS105). (c) Release of IFN-γ in co-cultures of emigrants with either syngeneic or allogeneic T cells, measured in a standard sandwich enzyme-linked immunosorbent assay (ELISA)

Figure 4

Maturation of esDCs (a) High-power phase-contrast micrograph of esDCs cultured overnight in LPS, showing acquisition of many dendrites and veils of cytoplasm The inset shows untreated esDCs for comparison The scale bar represents 10 μm (b) Flow cytometric analysis of mature esDCs (filled histograms) Open histograms represent the levels of background staining obtained with species- and isotype-matched monoclonal antibodies (c) Enhanced stimulation of the allogeneic MLR by mature esDCs (filled circles) compared with immature emigrants (open circles) measured on days 2–3 of the culture period, prior to the acquisition of significant immunostimulatory activity by untreated esDCs (see Figure 3a) Syngeneic responses elicited by mature esDCs (open triangles) and immature emigrants (filled triangles) are included for comparison



allogeneic T cells, compared with syngeneic controls, the response peaking on day 4 of culture. Significantly, proliferative responses were partially inhibited by non-depleting CD4 and CD8 monoclonal antibodies when added singly, and were completely abrogated by a combination of the two (Figure 3b), suggesting that both class I-restricted cytotoxic T lymphocytes (CTL) and class II-restricted Th cells actively contribute to this response. These findings unequivocally identify emigrants as belonging to the DC lineage: consequently, these cells have been designated ES cell-derived DCs (esDCs) to reflect their lineage commitment.

In accordance with the results of RT–PCR analysis (Figure 1b), supernatants from cultures containing allogeneic, but not syngeneic, T cells contained 500–700 pg/ml bioactive IL-12, while up to 1200 pg/ml IL-18 could be detected throughout, suggesting its constitutive secretion. As both cytokines contribute to the polarisation of responding cells towards a Th1 phenotype, we measured the release of interferon-γ (IFN-γ) and IL-4. As anticipated, high levels of IFN-γ were evident in supernatants from allogeneic cultures, whereas syngeneic T cells released significantly less (Figure 3c). IL-4, by contrast, could not be detected in either setting (data not shown). Although the stimulation of a potent Th1 response is reminiscent of the lymphoid-related subset of murine DCs [10], the absolute dependence of esDCs on GM-CSF and their lack of expression of CD8α and Dec-205 (data not shown) strongly favour a myeloid origin. These findings add weight to suggestions that the ability to elicit a Th1 response is not a property confined to particular DC subsets, but is influenced, instead, by environmental factors [11].

To determine whether esDCs might be induced to mature in a co-ordinated fashion, cells were harvested and replated for 3 days, 1 μg/ml lipopolysaccharide (LPS) being added for the final 24 hours. Following exposure to LPS, these cells acquired a highly dendritic appearance (Figure 4a), with more prominent veils of cytoplasm than their immature counterparts (Figure 4a, inset). Flow cytometry revealed the appearance of the DC-restricted marker CD11c and upregulation of MHC class II and the co-stimulatory molecules CD40, CD54, CD80 and CD86 (Figure 4b). Consistent with their phenotype, mature esDCs elicited greatly enhanced responses among allogeneic T cells (Figure 4c), frequently stimulating proliferation above background levels at densities of 300 cells per well, such responses peaking 24–36 hours earlier than those stimulated by their immature counterparts.

The derivation of long-term cultures of DCs from pluripotent ES cells has implications for our understanding of DC ontogeny and function. The requirement for IL-3 to secure the development of esDCs from EBs, but the absence of IL-3Rα chain expression by the resulting emigrants (data not shown), strongly suggests esDC development from an early IL-3-dependent hematopoietic progenitor, similar to

that described in humans [12]. Significantly, the lack of requirement for exogenous IL-3 for the development of DCs from bone marrow suggests that esDCs represent a population more primitive than previously described, a possibility supported by the lack of spontaneous maturation commonly observed among bmDCs: esDCs maintained continuously in culture for 4 weeks without harvesting showed no detectable changes in surface phenotype or stimulatory capacity. The evident stability of their phenotype over time, but their ability, nevertheless, to respond to maturation stimuli, make this novel source of DC particularly amenable to exploitation experimentally, as does their potential to express heterologous genes introduced at the ES cell stage. Indeed, the development of protocols for the production of ES cells in which both alleles of a target gene have been disrupted [13] raises prospects for the production of stable lines of 'knockout' DCs for elucidating gene function. Furthermore, the recent description of pluripotent ES cells derived from human blastocysts [14], holds promise for the application of our approach to the identification of novel targets for immune intervention in human disease.

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Supplementary material

Supplementary material including additional methodological detail and data implicating GM-CSF in the expansion of esDCs is available at http://current-biology.com/supmat/supmatin.htm

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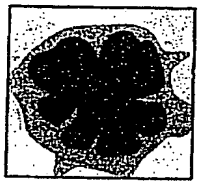

In vitro expansion of CD34⁺/CD41⁺ cells from human peripheral blood CD34⁺/CD41⁻ cells: Role of cytokines for in vitro proliferation and differentiation of megakaryocytic progenitors

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Abstract

The aim of this study is to clarify the transitional change of the proliferation and differentiation of human peripheral blood CD34⁺ cells to megakaryocytic lineage, focusing on its clinical application. We developed a rapid system to purify human peripheral blood CD34⁺ cells from healthy volunteers, which produced CD34⁺ cells with a 90% purity. The purified CD34⁺ cells predominantly consisted of CD41⁻ cells, and the rate of coexpression of CD41 was 0.6% ± 0.5%. When the purified cells were cultured in liquid phase for 10 days in the presence of recombinant human stem cell factor (rSCF; a ligand for c-kit), interleukin-3 (rIL-3), and thrombopoietin (rTPO; a ligand for Mpl), the number of CD34⁻/CD41⁺ cells increased to 19% ± 7% of total expanded cells on day 4 (4 days of liquid culture) and then gradually decreased to 2.2% ± 0.6% on day 10. The absolute number of CD34⁺/CD41⁺ cells increased and reached a plateau on day 6, and $1.7 \pm 0.6 \times 10^5$ CD34⁺/CD41⁺ cells were produced by 1×10^5 CD34⁺/CD41⁻ day 0 cells. The CD34⁻/CD41⁻ cells appeared on day 6, continuously increased in number until day 10, and constituted the main population of expanded cells on day 10, with a value of 38% ± 18%. On day 10, $19.5 \pm 10.6 \times 10^5$ of CD34⁻/CD41⁺ cells were produced by 1×10^5 CD34⁺/CD41⁻ day 0 cells. The deletion of rTPO from this cytokine combination decreased the number of CD34⁺/CD41⁺ and CD34⁻/CD41⁺ cells, after days 6 and 8, respectively. Day 0 cells required rIL-3 for promoting colonies containing megakaryocytes, whereas rTPO alone promoted almost no megakaryocytic colonies from day 0 cells. Thus, a combination of IL-3 and SCF expands CD34⁺/CD41⁺ cells from CD34⁺/CD41⁻ cells, and TPO mainly acts to increase CD34⁻/CD41⁺ cells. This study suggests that if the expansion of CD34⁺/CD41⁺ is performed in vitro, the 6 days' culture of peripheral blood CD34⁺/CD41⁻ cells with a combination of IL-3 and SCF with TPO provides the most rapid and stable products of CD34⁺/CD41⁻ cells for the rapid recovery of platelets in patients with peripheral blood stem cell transplantation.

Introduction

Numerous cytokines, although not specific for megakaryocytopoiesis, stimulate megakaryocytic differentiation. Interleukin-3 (IL-3) and granulocyte-macrophage colony-stimulating factor (GM-CSF) promote the proliferation and differentiation of megakaryocytes [1,2]. Interleukin-6 primarily stimulates megakaryocytic differentiation [3], whereas interleukin-11 increases the size and ploidy of megakaryocytes [4]. Stem cell factor (SCF; a ligand for c-kit) by itself has no effect but does potentiate the ability of other cytokines to stimulate megakaryocytic colony formation [5]. The thrombopoietin (TPO; a ligand for Mpl) exerts a stimulatory activity, in vivo and in vitro, restricted to the megakaryocytic lineage [6–12] and stimulates human hematopoietic progenitor cells to generate megakaryocytic colonies in semisolid medium and to generate a progeny of megakaryocytes in liquid culture, accompanied by the expression of glycoproteins GpIb and GpIIb (CD41)/IIIa (CD61) [3,12].

Several studies have shown that the flow cytometric measurement of CD34⁺ cells can be used to establish the reconstitutive capacity of a stem cell transplant [13–16]. In these studies, the reinfusion of peripheral blood stem cells (PBSCs) resulted in rapid granulocyte reconstitution in almost all patients but not always a rapid withdrawal from platelet transfusions. Current evidence indicates that GpIIb and GpIIIa are detected first during megakaryocytic differentiation [8,12,17–20]. The number of CD34⁺ cells expressing GpIIb or IIIa present in PBSC transplants correlates with platelet recovery after intensive chemotherapy. Dercksen et al. [21] found that patients who received more than the threshold of 0.34×10^6 CD34⁺/CD41⁺ cells/kg had a significantly shorter time to platelet recovery compared with the patients who received fewer CD34⁺/CD41⁺ cells and that CD34⁺/CD41⁻ cells correlated substantially better with the time to platelet recovery after autologous PBSC transplantation than did the total number of CD34⁺ cells. This observation suggests that the CD34⁺/CD41⁺ cells represent megakaryocytic precursors that are responsible for platelet recovery for PBSC transplantation.

This study focused on the CD34⁺/CD41⁻ cell population, which is assumed to be a highly selected population of noncommitted progenitors to megakaryocytic lineage. Platelets can bind to CD34⁺ cells from human blood and bone marrow, and this interaction interferes with the accurate detection of

endogenously expressed platelet glycoproteins Under conditions that prevent platelet adhesion, a small subpopulation of CD34+ cells expresses the platelet GpIIb-IIIa complex [21]. We developed a rapid system to purify human peripheral blood CD34+ cells from healthy volunteers, which produced CD34+ cells with a 90% purity [22]. The purified CD34+ cells consisted predominantly of CD41- cells, and the rate of coexpression of CD41 was low at 0.6% ± 0.5%, indicating that it is feasible to investigate a time course of megakaryocytic proliferation and differentiation.

The aims of this study were to evaluate the transitional change of the expression rate, expansion, and the developmental state of megakaryocytic progenitors, using flow cytometry and in vitro colony assay, to investigate the role of TPO, IL-3, and SCF for expanding CD34+/CD41+ and CD34-/CD41+ cells, focusing on clinical application.

Materials and methods

Blood specimens

Peripheral blood was obtained, with informed consent, from healthy adult Japanese volunteers, and approximately 400 mL of peripheral blood was collected in an acid citrate dextrose-A (ACD-A) solution (ACD-A; Terumo, Tokyo, Japan) at a final concentration of 13% (weight/volume%)

Separation of mononuclear cells and depletion of platelets

The peripheral blood cells were separated over Ficoll-Hypaque (1.077 g/cm^3; Pharmacia Fine Chemicals, Piscataway, New Jersey) at 400*g* for 25 minutes at 24°C. The interface mononuclear Ficoll-Hypaque cells were collected, washed twice with Ca^{2+}-, Mg^{2+}-free Dulbecco's phosphate-buffered saline (PBS) containing 1% ACD-A and 0.3% deionized bovine serum albumin (D-BSA; Sigma Chemical, St Louis, Missouri [DAD-PBS]), and resuspended in 10 mL of DAD-PBS. The cell suspension was overlaid on 5 mL of 10% BSA in PBS containing 1% ACD-A in a 15-mL conical tube (Becton-Dickinson, Lincoln Park, New Jersey) and was then centrifuged at 180*g* for 10 minutes at 24°C to remove platelets; this procedure was then repeated. The cells were washed once with DAD-PBS and resuspended in 10 mL of Iscove's modified Dulbecco's medium (IMDM; Sigma) containing 0.3% D-BSA (IMDM-BSA) (mononuclear cells)

Depletion of adherent cells using nylon fiber

The mononuclear cells in 10 mL of IMDM-BSA were incubated with nylon fiber (Wake Pure Chemical Industries, Osaka, Japan) to deplete adherent cells by modification of a method described by Julius et al [23]. The procedure has been described in detail elsewhere [22]. In brief, we used a nylon fiber syringe instead of a nylon fiber column. After nylon fiber treatment, the nonadherent cells were collected and centrifuged at 400*g* for 5 minutes at 4°C and resuspended in IMDM-BSA at a concentration of 2×10^7/mL (nylon fiber cells)

Purification of CD34+ cells using immunomagnetic microspheres

The method used has been described in detail elsewhere [22,24]. In brief, the nylon fiber cells were incubated with 2.5 μg/mL anti-CD34+ monoclonal antibody (mAb) (QBEND10; Seikagaku, Tokyo, Japan), which detects an epitope sensitive to chymopapain. The cell suspension was incubated at 3°C, using an end-over-end rotator (RVM-100; Iwaki Glass, Funabashi, Japan). After 30 minutes of incubation, the cells were washed twice with cold IMDM-BSA and resuspended in IMDM-BSA at a concentration of 2×10^7/mL in a 12×75-mm polystyrene tube (Becton-Dickinson). Incubation with immunomagnetic microspheres (Dynabeads M-450 coated with goat antimouse IgG, Dynal, Great Neck, New York) was done at a 1:5 ratio of microspheres per cell at 4°C using an end-over-end rotator. After 30 minutes, the cell-bound microspheres and free microspheres attached themselves to the tube wall (Dynal MPC-1, Dynal, Fort Lee, New Jersey), and the free cells that did not bind microspheres were removed. The tube then was disconnected from the magnet, and the microspheres were washed by gentle pipetting with 3 mL of cooled IMDM-BSA. The microspheres were again attached to the tube wall using the magnet, and cells released by washing were removed (microsphere washing procedure). The microsphere washing procedure was repeated twice with cooled IMDM-BSA and twice more with cooled IMDM to remove BSA. The cells that bound microspheres and free microspheres were then gently pipetted 10 times with 2 mL of IMDM containing 130 U/mL of chymopapain (Chymodiactin, Knell Pharmaceuticals, Nottingham, England), left for 10 minutes at room temperature (24°C), and pipetted 10 times every 5 minutes. The tube was then attached to the magnet for 2 minutes at 24°C. The cells released from the microspheres were collected as the CD34+ fraction. This fraction was immediately mixed with 4.5 mL of heat-inactivated fetal calf serum (FCS) (Flow Laboratories, McLean, Virginia) at 24°C in a 15-mL conical tube to dilute the chymopapain activity. The microspheres were washed once with 3 mL of IMDM-BSA and reattached to the magnet, and the remaining cells released from the microspheres were collected and pooled with the CD34- fraction. The cells were centrifuged at 400*g* for 5 minutes at 4°C and resuspended in 3 mL of IMDM-BSA in a 15×75-mm polystyrene tube. The tube was again attached to the magnet to remove contaminated microspheres, and cells free from the microspheres were collected into a 15-mL conical tube. The cells were then centrifuged at 400*g* for 5 minutes at 4°C and resuspended in IMDM-BSA in a small volume to count the cells (approximately 0.4 mL) (CD34+ cells).

Flow cytometry

Phenotyping of the purified cells was analyzed by flow cytometry (Ortho Cytoron, OrthoDiagnostic Systems, Raritan, New Jersey), as reported previously [22,25]. The following reagents were used: hematopoietic progenitor cell A-2 (8G12, which recognizes chymopapain-resistant CD34+ epitopes: fluorescein isothiocyanate conjugate [FITC] or phycoerythrin conjugate [PE] Becton Dickinson), Leu17 (CD38, PE, Becton Dickinson), My9 (CD33, PE, Coulter Immunology, Hialeah, Florida), OKDR (HLA-DR, PE, OrthoDiagnostic), OKT3 (CD3, PE, OrthoDiagnostic), My7 (CD13, PE, Coulter Immunology), My4 (CD14, PE, Coulter Immunology), B4 (CD19, PE, Coulter Immunology), and TP80 (CD41, FITC, Nichirei, Tokyo, Japan). For each specimen, 5000 to 20,000 cells were counted.

Subsets that were CD34+/CD41-, CD34+/CD41+, or CD34-/CD41+ were sorted under sterile conditions using appropriate positive and negative controls by fluorescence-activated cell sorting (FACSvantage; Becton Dickinson, Mountain View, Virginia) run by Lysis II. Purified subsets were grown in fibrin clot and in suspension cultures using the combinations of cytokines described in the text.

Liquid suspension culture of CD34+ cells

The purified CD34+ cells were cultured in a liquid phase, as described elsewhere [26]. In brief, the purified cells ranging from $3–5\times10^3$ cells/mL were suspended in a 1-mL mixture containing 20% FCS, 10% heat-inactivated pooled human AB serum, 1% BSA, 10-μg/mL insulin (porcine sodium, activity 26.3 United States Pharmacopoeia U/mg; Calbiochem, Behring Diagnostics, La Jolla, California), with or without saturating doses of rIL-3 (10^4 chronic myelogenous leukemia U/mg; AmGen Biologicals, Thousand Oaks, California) at 100 U/mL, recombinant stem cell factor (rSCF; Kirin Brewery, Tokyo, Japan) at 100 ng/mL, and thrombopoietin (TPO; Kirin) at 100 ng/mL, in the presence of 5×10^{-5}M 2-mercaptoethanol (Sigma), penicillin at 50 U/mL, streptomycin at 50 μg/mL (Flow Laboratories), and IMDM in 12×75-mm polystyrene tubes (Becton-Dickinson). After incubation for the indicated periods at 37°C in 5% CO_2-95% atmosphere, the cells were collected every other day, washed twice with IMDM containing 0.25% BSA, and stored at 3°C until use

Table 1. Effect of chymopapain treatment on CD41 epitopes

Exp. no	Chymopapain treatment (CD41 expression, %) (–)	(+)
1	8.1	8.3
2	10.1	11.1
3	9.9	9.1
Mean ± SD	9.4 ± 1.1	9.5 ± 1.4

Day 0 purified PB CD34⁺ cells were incubated in liquid culture with rSCF, rIL-3 and rTPO, as described under Methods. After 4 days of culture, the cells were collected, washed twice and incubated with IMDM or IMDM containing 130 U/ml chymopapain for 10 min at room temperature. The cells were centrifuged, washed twice and analyzed by flow cytometry, as described under Methods.

Semisolid culture of progenitors

The purified cells (CD34⁺ cells) and those after liquid culture were incubated in triplicate at a concentration of 300 cells/mL for the CD34⁺ cells and at concentrations ranging from 3×10^2 to 1×10^4 cells/ml for the expanded cells, in flat-bottomed, 24-well, tissue culture plates (Linbro, Flow Laboratories) with 0.5-mL serum containing fibrin clots, as described [24], with or without rSCF at 50 ng/mL, rIL-3 at 50 U/mL, and rTPO at 100 ng/mL. Based on the time course study of megakaryocytic colony formation by TPO and TPO+SCF+rIL-3, the clots were incubated until day 14, including the period in liquid phase, at 37°C in a 5% CO_2-95% atmosphere and were fixed and stained by an avidin-biotin indirect-immunoperoxidase method [27] or with May-Günwald-Giemsa stain.

Differences between groups of data were analyzed with the Wilcoxon-Mann-Whitney U test, with $p < 0.05$ taken as a significant difference.

Results

Characteristics of purified CD34⁺ cells

The purity of CD34⁺ cells in the purified cells was 89.8% ± 3.5% as a mean ± SD of seven separate experiments. Subpopulations of the CD34⁺ cells purified by this method have been reported elsewhere [22]. In brief, the CD34⁺ cells mainly consisted of CD38⁺ cells (98.6% ± 0.8%) and HLA-DR⁻ cells (95.5% ± 3.6%). The rate of coexpression of CD117 (c-kit) and CD13 was 8.8% ± 2.9% and 42.3% ± 14.8%, respectively, and that of CD41 was low at 0.6% ± 0.5% and then increased thereafter, depending on the culture conditions, as described in the following section. The low rate of coexpression of CD41 could be the result of damage to the CD41 epitopes by the chymopapain used in purifying the CD34⁺ cells. Further experiments showed, however, that chymopapain treatment of day 6 cells, which were incubated with rSCF, rIL-3, and rTPO for six days and expressed CD41 epitopes for 10 minutes did not reduce the rate of coexpression of CD41 (Table 1). Thus, the CD34⁺ cells derived from steady-state peripheral blood predominantly consist of CD34⁺/CD41⁺ cells.

Generation of total cells and CD34⁺ cells

When the purified CD34⁺ cells were incubated with rSCF, rIL-3, and rTPO for 10 days in liquid medium, the total number of cells continuously increased and reached a maximum on day 10, with a value of 47.7 ± 17.1-fold (Table 2), and showed morphologic or phenotypic differentiation toward megakaryocytes (Figs. 1 and 2). There was no statistical significance in the expansion rate of total cells, with or without rTPO (Table 2). The positive rate of CD34 in the expanded cells remained unchanged until day 6, then rapidly decreased, as shown in Table 2. Because the total number of cells continuously increased, the number of CD34⁺ cells continuously increased until day 6, reaching a maximum value of 12.5 ± 3.2-fold. There was no statistical significance in the expansion rate of CD34⁺ cells, with or without rTPO (Table 2).

Expression and generation of CD34⁺/CD41⁺ cells

Figures 3A and 3B show the change of the expression rates of $CD34^+/CD41^+$ and $CD34^-/CD41^+$ cells, respectively, in the total cell population. When the purified $CD34^+/CD41^-$ cells were cultured in liquid phase with rSCF, rIL-3, and rTPO (Figure 3A), the $CD34^+/CD41^+$ cells appeared until day 2 and consisted of 15% ± 3% of total cells. The maximum expression rate was noted on day 4 with a value of 19% ± 7%, but with no statistical significance compared with that of day 2 or 6. At day 4, the CD41⁺ cells consisted predominantly of $CD34^+/CD41^+$ cells with a value of 96%. The expression rate of $CD34^+/CD41^+$ cells then continuously decreased to 2.2% ± 0.6% until day 10. Deletion of rTPO from this cytokine combination seemed to decrease the expression rate of $CD34^+/CD41^+$ cells after day 6, but with no statistical significance. In addition, to exclude the

Table 2. Expansion of CD34⁺ cells

Days of culture/Factors	Total cells (fold) SCF+IL-3	SCF+IL-3+TPO	CD34 expression rates (%) SCF+IL-3	SCF+IL-3+TPO	CD34⁺ cells (fold) SCF+IL-3	SCF+IL-3+TPO
0	1	1	89.1 ± 4.2	90.6 ± 3.6	1	1
2	1.7 ± 1.0	1.2 ± 0.2	94.4 ± 3.3	83.5 ± 16.2	1.8 ± 1.2	1.2 ± 0.4
4	3.6 ± 0.6	3.1 ± 0.6	95.4 ± 1.5	93.8 ± 1.6	3.9 ± 0.7	4.3 ± 1.6
6	11.6 ± 4.2	14.5 ± 3.9	75.6 ± 6.1	78.1 ± 3.4	9.8 ± 3.3	12.5 ± 3.2
8	29.2 ± 11.0	34.9 ± 8.0	17.0 ± 10.1	14.0 ± 7.3	5.4 ± 3.7	5.2 ± 2.7
10	43.2 ± 15.0	47.7 ± 17.1	3.7 ± 1.7	5.4 ± 1.9	1.7 ± 0.6	2.8 ± 1.1

Effects of two different combinations of cytokines on proliferation of total cells, CD34⁺ cells and CD34 expression rate of the purified cells from peripheral blood. The purified cells were incubated in liquid phase, as described under Methods. The mean values for 4 separate experiments ± SD are shown.

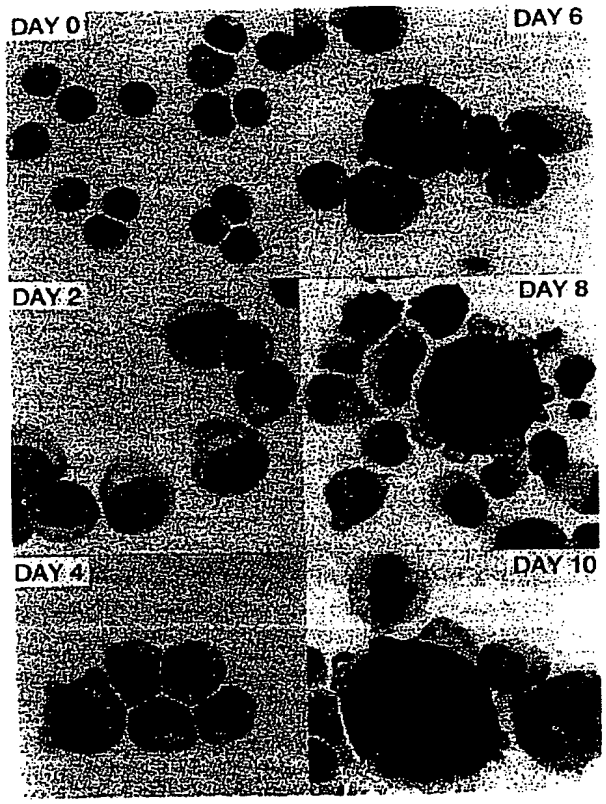

Fig. 1. Transitional change in morphology of the purified CD34+ cells in liquid culture. The purified cells (day 0) that consisted of 99.0% of CD34+ cells were incubated in liquid culture for 10 days in the presence of rTPO, rSCF, and rIL-3. At the indicated days, the cells were collected and washed twice, and cytocentrifuge preparations were made. The cells were stained with May-Grünward-Giemsa stain

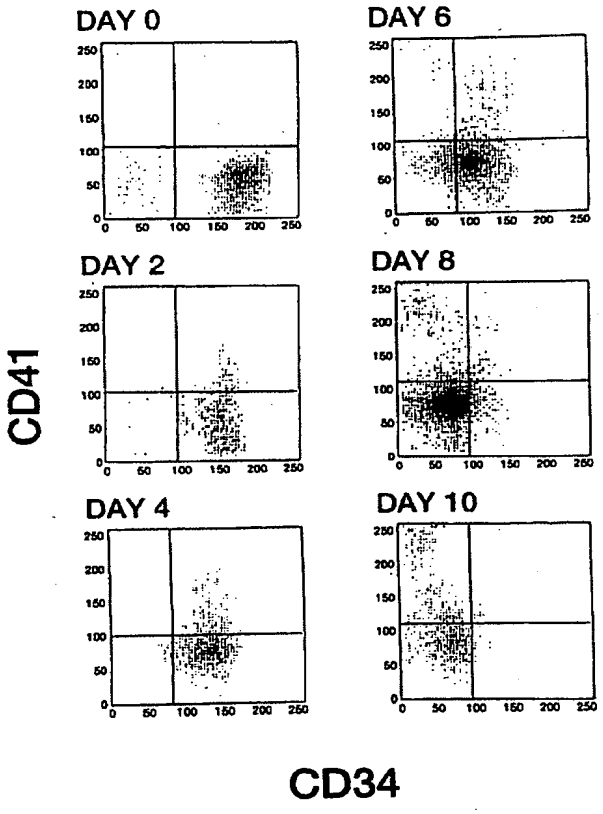

Fig. 2. Membrane phenotype of purified cells grown in liquid culture in the presence of rTPO, rSCF, and rIL-3 is shown. Cells were harvested at days 0, 2, 4, 6, 8, and 10 and were analyzed by double fluorescence labeling using anti-CD34/anti-CD41 MoAbs. A representative experiment is shown

possibility that the expanded CD34+/CD41+ cells were derived from CD34+/CD41− cells present in the purified CD34+ cells, CD34+/CD41− cells were sorted from day 0 CD34+ cells. When day 0 CD34+/CD41− cells were cultured in liquid phase with rSCF, rIL-3, and rTPO, the CD34+/CD41+ cells were generated and consisted of 99.9% of total cells after 6 days of culture.

Because a subset of CD41+ cells in day 0 CD34+ cells was small and varied widely from 0.1% to 1.3%, it was considered inappropriate to use "expansion rate" to express the grade of amplification of CD41+ subsets. Therefore, the absolute number of expanded CD41+ subsets is shown in Table 3 as the number generated from 1×10^5 day 0 CD34+ cells. In the presence of rSCF, rIL-3, and rTPO, 1×10^5 day 0 CD34+ cells generated CD34+/CD41+ cells with a maximum value of $1.7\times10^5 \pm 0.6\times10^5$ cells on day 6. The number of these cells appeared to decrease after days 8 and 10, but with no statistical significance compared with that of day 6. The deletion of rTPO from this cytokine combination decreased the number of CD34+/CD41− cells after day 6, with a statistical significance on days 6 and 8.

Expression and generation of CD34−/CD41+ cells

When the purified CD34+ cells were cultured in liquid phase with rSCF, rIL-3, and rTPO, the first increase of CD34−/CD41+ cells was noted on day 6 and consisted of 3.1% ± 1.7% of total cells (Fig. 3A and Table 3). The expression rate of these cells continued to increase and reached a maximum value of 38% ± 18% on day 10. When rTPO was removed from this cytokine combination, the expression rate of CD34−/CD41+ cells significantly decreased compared with that with rTPO on days 8 (5% ± 3% without TPO vs. 15% ± 7% with TPO; $p < 0.05$) and 10 (11% ± 8% without TPO vs. 38% ± 18% with TPO; $p < 0.05$).

In the presence of rSCF, rIL-3, and rTPO, the first increase of CD34−/CD41− cells was noted on day 6, and 10 days' liquid culture of 1×10^5 day 0 CD34+ cells that contained 450 ± 370 CD34−/CD41+ cells generated $19.6 \pm 10.5\times10^5$ CD34−/CD41+ cells (Table 3). When rTPO was removed from this cytokine combination, the number of CD34−/CD41− cells generated significantly decreased compared with that with rTPO on days

Table 3. Expansion of CD41⁺ subsets

Days of culture/Factors	Theoretical No. of CD41⁺ cells that can be expanded from 1×10⁵ PB CD34⁺ cells			
	CD34⁺/CD41⁺ cells (×10⁵)		CD34⁻/CD41⁺ cells (×10⁵)	
	SCF+IL-3	SCF+IL-3+TPO	SCF+IL-3	SCF+IL-3+TPO
0	0 009 ± 0.006	0 005±0 005	0 009 ± 0.005	0.005 ± 0.004
2	0.425 ± 0.259	0.203 ± 0 059†	0 005 ± 0 004	0.029 ± 0 031
4	1 286 ± 0.642	0 936 ± 0.566†	0 020 ± 0 010	0.039 ± 0.029
6	0 527 ± 0.254†	1 677 ± 0 566*†	0 353 ± 0 154†	0 534 ± 0 309†
8	0.870 ± 0 623†	1 309 ± 0 635†	1 446 ± 0.362†	5.638 ± 3 330*†
10	0.532 ± 0 208†	1.109 ± 0 215*†	4 952 ± 2.802†	19.507 ± 10.516*†

The purified cells (day 0) were incubated in liquid culture for 10 days with rSCF and rIL-3, with or without rTPO at concentrations ranging from 3,000 to 50,000 cells/mL. At the indicated days, the cells were collected, washed twice and expression rates of CD41⁺ subsets in CD34⁺ cells or CD34⁻ cells were examined, as described under Methods. The values of each subset were expressed as theoretical numbers that can be generated from 1×10⁵ day 0 CD34⁺ cells and 4 individual experiments ± SD are shown. The experiments parallel data shown in Figure 2. *$p < 0.05$ as compared to the value with rSCF and rIL-3. †$p < 0.05$ as compared to the value on day 0.

8 (1.4 ± 0.4×10⁵ without TPO vs. 5.6 ± 3.3×10⁵ with TPO; $p < 0.05$) and 10 (5.0 ± 2.8×10⁵ without TPO vs. 19.5 ± 10.5×10⁵ with TPO; $p < 0.05$).

Transitional change of CSFs requirement for megakaryocytic progenitors

To investigate the transitional requirement of CSFs for megakaryocytic colony expression, cells that were incubated in liquid culture with rSCF, rIL-3, and rTPO were replated into fibrin clots in the presence of rTPO with or without CSFs and cultured until day 14. As shown in Figure 4, by 8 days of incubation, the rate of megakaryocytic colony expression of the cells continuing in culture with rTPO alone reached a maximum that was equivalent to the rate observed for those stimulated by rTPO, rSCF, and rIL-3. The combination of rTPO and rSCF showed a curve almost identical to that observed with rTPO alone. When rTPO and rIL-3 were present, there was no apparent effect of a further addition of rSCF in megakaryocytic colony expression.

To compare the proliferative capacity of megakaryocytic progenitor cells cultured with rTPO, rIL-3, and rSCF, we ascertained the size distributions of megakaryocytic colonies derived from liquid cultured cells in the presence of rTPO, rSCF, and rIL-3, as shown in Figure 5. When the purified CD34⁺ cells were incubated in liquid culture for six days (day 6 cells) and replated into fibrin clots, the number of megakaryocyte colonies that contained more than 51 megakaryocytes dramatically decreased. A peak value of megakaryocytic colonies that contained 10 to 50 megakaryocytes was observed on day 4, whereas a peak of the smallest megakaryocytic colonies that contained 3 to 9 megakaryocytes was observed on day 8. Therefore, the proliferative capacity of megakaryocyte colonies diminishes with an increase in incubation time in the liquid culture.

To confirm that megakaryocytic progenitors are present in a fraction of CD34⁺/CD41⁺ cells expanded with rSCF, rIL-3, and rTPO, day 0 CD34⁺ cells were cultured in liquid phase with rSCF, IL-3, and rTPO for six days, and subsets that were CD34⁺/CD41⁺ or CD34⁻/CD41⁺ were sorted and grown in fibrin clot using the combinations of growth factors. As shown in Table 4, when CD34⁺/CD41⁺ cells were cultured with cytokine combinations the included rIL-3, these cells formed megakaryocytic colonies with a maximum number compared with those seen without rIL-3. CD34⁻/CD41⁺ cells persisted as a single cell in the clot or formed small megakaryocytic colonies. There was no difference between rIL-3 and rTPO in the effect of promoting megakaryocytic colonies by CD34⁻/CD41⁺ cells.

Discussion

Platelet interaction with CD34⁺ cells is mediated by P-selectin [21]. Treatment with the proteolytic enzyme trypsin partially

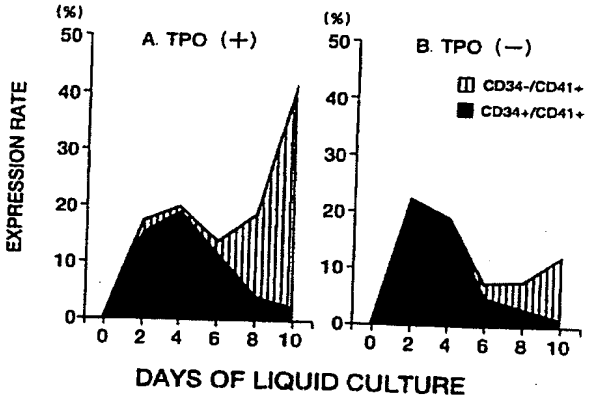

Fig. 3. Transitional change of expression rate of CD41⁺ subsets. The purified cells (day 0) were incubated in liquid culture for 10 days with rSCF and rIL-3 with **(A)** or without **(B)** rTPO. At the indicated days, the cells were collected and washed twice, and expression rates of CD34⁺/CD41⁺ and CD34⁻/CD41⁺ cells were examined, as described under Methods. The mean ± SD for four individual experiments is shown. The purity of CD41⁺ cells in day 0 cells was 0.6% ± 0.5%.

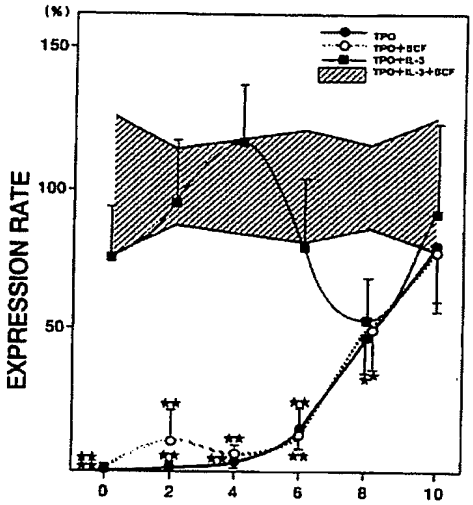

Fig. 4. Transitional change of CSFs requirement for generated cells in megakaryocytic development. The purified day 0 cells that consisted of 91% ± 4% CD34⁺ cells were incubated in liquid culture for 10 days with rTPO, rSCF, and rIL-3. At the indicated days, the cells were collected, washed twice, and plated into fibrin clots with rTPO alone (●), with rTPO plus rSCF (○), with rTPO plus rIL-3 (■), and with rTPO plus rSCF plus rIL-3 (shaded area) at concentrations of 500 to 6000 cells/mL. After incubation of the plated cells until day 14, the clots were fixed and stained, and the colonies that gave rise to 3 or more megakaryocytes were counted as CFU megakaryocyte colonies. Each point represents the mean value ± SE for four separate experiments and is expressed as a percentage of the value obtained in the presence of rTPO, rSCF, and rIL-3 (shaded area). $^{*}p < 0.05$ and $^{**}p < 0.01$ compared with the value with rTPO, rSCF, and rIL-3.

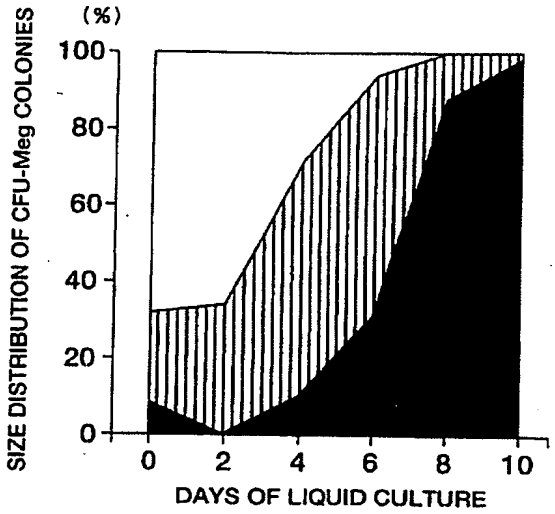

Fig. 5. Size distribution of CFU-Meg colonies. The purified day 0 cells that consisted of 91% ± 4% CD34⁺ cells were incubated in liquid culture for 10 days with rTPO, rSCF and rIL-3. At the indicated days, the cells were collected, washed twice, and plated into fibrin clots with rTPO, rSCF, and rIL-3 at concentrations of 500 to 6000 cells/mL. After incubation of the plated cells until day 14, the clots were fixed and stained, and colonies that gave rise to 3–9 (closed area), to 10–50 (hatched area), or to 51 or more megakaryocytes (open area) were counted. Each area represents the mean percentage of total CFU-Meg colonies for four separate experiments. The expression rate of total CFU-Meg colonies by the cells was 7.1% ± 2.4% for day 0, 6.2% ± 1.9% for day 2, 7.6% ± 1.9% for day 4, 6.3% ± 0.9% for day 6, 8.0% ± 1.1% for day 8, and 5.3% ± 1.7% for day 10.

inhibits the binding of activated platelets to CD34⁺ cells [21]. The immunomagnetic bead separation technique followed by chymopapain treatment to detach cells from the beads also releases most platelet fragments from the CD34⁺ cells [21,28]. The method we developed for the purification of peripheral blood CD34⁺ cells includes the inhibition of platelet aggregation by ACD-A solution, repeated platelet depletion by 10% BSA density gradient, monocyte/macrophage depletion by nylon fiber, and chymopapain treatment. These steps abolished the nonspecific binding of CD41 mAbs to the purified CD34⁺ cells and allowed for a direct study of the CD34⁺/CD41⁻ cell population.

The present study provides insight into early megakaryocytopoiesis. CD41, a specific platelet glycoprotein, is nearly absent in steady-state peripheral blood CD34⁺ cells. When peripheral blood CD34⁺ cells that predominantly consisted of CD34⁺/CD41⁻ cells were cultured in liquid phase for 10 days in the presence of rSCF, rIL-3, and rTPO, these cells produced CD34⁺/CD41⁺ subset and the CD34⁻/CD41⁺ subset thereafter. The CD34⁺/CD41⁻ cells increased to 19% ± 7% on day 4 and then gradually decreased to 2.2% ± 0.6% on day 10. The absolute number of CD34⁺/CD41⁺ cells generated from 1×10^5 day 0 CD34⁺/CD41⁻ cells that contained 450 ± 506 CD34⁺/CD41⁺ cells reached a plateau on day 6, with a value of $1.7 \pm 0.6\times10^5$ cells. Interestingly, the deletion of rTPO from this combination did not affect the expression rate or the expansion of CD34⁺/CD41⁺ cells until day 4, which indicates that rTPO is unnecessary to generate CD34⁺/CD41⁺ cells from peripheral blood CD34⁺/CD41⁻ cells. The reason rTPO combined with rSCF and rIL-3 supports a higher number of CD34⁺/CD41⁺ cells compared with that without rTPO after day 6 is unclear, but one explanation is that rTPO may maintain the survival (or prevent apoptosis) of a more mature compartment in a CD34⁺/CD41⁺ subset.

rTPO combined with rIL-3 and rSCF dramatically increased the number of CD34⁻/CD41⁺ cells after eight days of liquid culture compared with that without rTPO, which also indicates that rTPO mainly acts on mature megakaryocytic progenitors such as CD34⁺/CD41⁺ cells to expand CD34⁻/CD41⁻ cells. The difference of cytokine requirement depended on the maturation level of expanded cells. rIL-3 supported CD34⁺/CD41⁻ day 0 cells to promote colonies containing megakaryocytes, whereas rTPO alone had little effect in promoting colonies from these cells. Thus, a combination of rIL-3 and rSCF expanded CD34⁺/CD41⁺ cells

Table 4. CFU-Meg expression of CD41⁺ subsets

Growth factors	No. of CFU-Meg colonies/100cells	
	CD34⁺/CD41⁺	CD34⁻/CD41⁺
TPO	8 3 ± 2.3	3 7 ± 1.5
IL-3	34.0 ± 2.7	5.0 ± 5 3
SCF	0.7 ± 0.6	0.3 ± 0.6
IL-3+SCF	38 3 ± 2.9	0.7 ± 0 6
TPO+IL-3	33.7 ± 5 9	1.7 ± 1.5
TPO+SCF	2.7 ± 1.2	2.0 ± 1.0
TPO+IL-3+SCF	46.0 ± 16.5	10.7 ± 5.0

Day 0 purified PB CD34⁺ cells were incubated in liquid culture with rSCF, rIL-3 and rTPO. After 6 days of culture, the cells were collected, washed twice and subsets of CD41⁺ cells were sorted, as described under Methods. Values are mean ± SD of triplicate culture

from CD34⁺/CD41⁻ cells, regardless of the presence or absence of rTPO. rTPO may act to sustain CD34⁺/CD41⁺ cells and to generate CD34⁻/CD41⁺ cells from CD34⁺/CD41⁺ cells.

Based on evidence that a rapid platelet recovery after PBSC transplantation can be achieved by more than 3.4×10^5 CD34⁺/CD41⁺ cells/kg [21], it would be feasible to conduct in vitro expansion of CD34⁺/CD41⁺ cells from peripheral blood CD34⁺ cells for PBSC transplantation. For example, when 1×10^5 steady-state peripheral blood CD34⁺ cells were cultured in liquid phase for six days with rTPO, rSCF, and rIL-3, CD34⁺/CD41⁺ cells expanded to 1.7×10^5 cells. The absolute number of initial 120×10^5 peripheral blood CD34⁺ cells was calculated to be sufficient to achieve the expansion of CD34⁺/CD41⁺ cells necessary for rapid platelet recovery after PBSC transplantation in patients weighing 60 kg. The expression rate of CD41 antigen on CD34⁺ cells mobilized from patients given granulocyte-colony stimulating factor following chemotherapy has been reported to be 6 3% (range 0%–18 1%) [21]. Of particular interest is the proposition that the pretreatment of donor mice with rTPO produces an increased enhancement of platelet recovery compared with the treatment of recipients with rTPO immediately after transplantation [29]. Although rTPO has synergistic effects in enhancing the proliferation of early hematopoietic progenitors [28], the predominant action is to increase the formation of mature megakaryocytes from CD34⁺/CD41⁺ cells [30]. Therefore, we estimate that recipients given an insufficient number of CD34⁺/CD41⁺ cells would derive little benefit from the in vivo administration of rTPO. In these patients, the combined use of nonexpanded PBSC and expanded CD34⁺/CD41⁺ cells would be one alternative, with or without the in vivo administration of rTPO, to make it more likely that the platelet count would normalize.

Acknowledgments

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Thrombopoietin, kit Ligand, and flk2/flt3 Ligand Together Induce Increased Numbers of Primitive Hematopoietic Progenitors From Human CD34$^+$Thy-1$^+$Lin$^-$ Cells With Preserved Ability to Engraft SCID-hu Bone

By Karin M. Luens, Marilyn A. Travis, Ben P. Chen, Beth L. Hill, Roland Scollay, and Lesley J. Murray

CD34$^+$Thy-1$^+$Lin$^-$ cells are enriched for primitive hematopoietic progenitor cells (PHP), as defined by the cobblestone area-forming cell (CAFC) assay, and for bone marrow (BM) repopulating hematopoietic stem cells (HSC), as defined by the in vivo SCID-hu bone assay. We evaluated the effects of different cytokine combinations on BM-derived PKH26-labeled CD34$^+$Thy-1$^+$Lin$^-$ cells in 6-day stroma-free cultures. Nearly all (>95%) of the CD34$^+$Thy-1$^+$Lin$^-$ cells divided by day 6 when cultured in thrombopoietin (TPO), c-kit ligand (KL), and flk2/flt3 ligand (FL). The resulting CD34^{hi} PKH^{lo} (postdivision) cell population retained a high CAFC frequency, a mean 3.2-fold increase of CAFC numbers, as well as a capacity for in vivo marrow repopulation similar to freshly isolated CD34$^+$Thy-1$^+$Lin$^-$ cells. Initial cell division of the majority of cells occurred between day 2 and day 4, with minimal loss of CD34 and Thy-1 expression. In contrast, cultures containing interleukin-3 (IL-3), IL-6, and leukemia inhibitory factor contained a mean of 75% of undivided cells at day 6. These CD34^{hi} PKH^{hi} cells retained a high frequency of CAFC, whereas the small population of CD34^{hi} PKH^{lo} postdivision cells contained a decreased frequency of CAFC. These data suggest that use of a combination of TPO, KL, and FL for short-term culture of CD34$^+$Thy-1$^+$Lin$^-$ cells increases the number of postdivision PHP, measured as CAFC, while preserving the capacity for in vivo engraftment.

PLURIPOTENT hematopoietic stem cells (HSC) are considered to be ideal targets for gene therapy. Use of retroviral vectors for gene transduction requires that the target cells pass through mitosis.[1,2] Because the majority of freshly isolated HSC are thought to be quiescent,[3] it is necessary to provide appropriate ex vivo conditions to stimulate HSC division without differentiation and subsequent loss of multilineage potential to achieve efficient clinical therapy with gene-manipulated HSC. Stroma appears to be required to provide such conditions,[4] but, due to the technical difficulties of using stromal cultures for clinical gene therapy trials, the appropriate culture conditions that stimulate ex vivo replication of human HSC in the absence of stroma must be defined.

To attempt retroviral gene transduction of HSC in the absence of stroma, interleukin-3 (IL-3) and IL-6 in combination with c-kit ligand (KL)[4] or leukemia inhibitory factor (LIF)[5] are usually added to stroma-free cultures. However, the efficiency of gene transduction into pluripotent HSC remains low. Ex vivo culture of HSC with IL-3 can be detrimental to maintenance of primitive HSC function, as was shown by decreased reconstituting ability of HSC in lethally irradiated mice.[6,7] Retrovirus-mediated gene expression in human hematopoietic cells correlated inversely with growth factor stimulation when cultures included IL-3.[8] In addition, IL-3 can abrogate B-lymphoid potential and is a positive regulator of early myelopoiesis.[9] We have previously shown that 3 to 6 days of culture in the presence of IL-3 induces not only cell division of primitive human CD34$^+$Lin$^-$Rhodamine (Rh123)^{lo} cells, but also differentiation (loss of CD34 expression).[10,11] There is now increasing evidence that inclusion of IL-3 in cultures results in loss of the long-term reconstituting ability of HSC.[6,7,12,13]

We, therefore, wished to investigate combinations of early acting stromal-derived cytokines for stimulation of CD34$^+$Thy-1$^+$Lin$^-$ cell division without differentiation. Signaling through tyrosine kinase receptors (TKR) is important to induce HSC division. The ligand for the TKR c-kit (kit ligand) plays an important role in stimulation of proliferation of HSC, usually in synergy with other cytokines.[14,15] Another important HSC-associated TKR, flk2/flt3, was first identified in the mouse.[16,17] Flk2/flt3 ligand stimulates proliferation of both murine[16,18-21] and human HSC.[18,19,22-24] In addition to these two factors, thrombopoietin (TPO), although originally believed to be a megakaryocyte (MK) lineage-specific cytokine,[25] has been shown to stimulate proliferation of HSC of both mouse[26-28] and human.[10,11,29-31]

The cobblestone area-forming cell (CAFC) assay allows in vitro estimation of the frequency of primitive hematopoietic progenitor cells (PHP) within a population, whereas the SCID-hu bone assay measures the in vivo bone marrow (BM) repopulating ability of HSC. Both CAFC and SCID-hu bone repopulating HSC are enriched among CD34$^+$Thy-1$^+$Lin$^-$ cells.[32-34] In the present study, we have compared different cytokine combinations added to short-term cultures of adult BM CD34$^+$Thy-1$^+$Lin$^-$ cells for retention of in vitro CAFC and in vivo SCID-hu bone repopulating ability within the population of divided human CD34^{hi} cells. One condition used for gene transduction, ie, IL-3, IL-6, and LIF, was compared with TPO plus KL[11] and with TPO, KL, plus flk2/flt3 ligand (FL).[35] Newly generated CD34^{hi} cells could be identified by the loss of the fluorescent membrane dye PKH26.[36-38] The optimal timepoint for maximal division with minimal differentiation was determined. TPO, KL, and FL in combination were found to stimulate division of a majority of CD34$^+$Thy-1$^+$Lin$^-$ cells by day 4, with minimal loss of CD34 or Thy-1 expression.

MATERIALS AND METHODS

Antibodies. To enrich for CD34$^+$Thy-1$^+$Lin$^-$ cells, we used Tuk3 (anti-CD34 obtained from Dr A. Ziegler, University of Berlin, Berlin, Germany) directly conjugated to sulphorhodamine (SR) and GM201 (antihuman Thy-1 from Dr W. Rettig, Ludwig Cancer Research

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Institute, New York, NY) directly conjugated to phycoerythrin (PE; SyStemix, Palo Alto, CA). As an isotype control for anti-CD34 (Tuk3) staining, we obtained FLOPC 21 mouse IgG_3 (Sigma, St Louis, MO) conjugated to SR (SyStemix). As a control for anti-Thy-1 staining, we used purified mouse IgG_1 (Becton Dickinson, Mountain View, CA) conjugated to PE (SyStemix). The lineage panel of fluorescein isothiocyanate (FITC)-conjugated antibodies Leu-5b (anti-CD2), Leu-M3 (anti-CD14), Leu-M1 (anti-CD15), Leu-11a (anti-CD16), SJ25C1 (anti-CD19). FITC-conjugated mouse IgG_1 and IgG_{2a}, PE- and FITC-conjugated HPCA-2 (anti-CD34), and PE-conjugated Leu-12 (anti-CD19) and Leu-M9 (anti-CD33) were purchased from Becton Dickinson. FITC-conjugated antibody D2.10 (antiglycophorin A) was purchased from AMAC (Westbrook, ME). Hybridomas that produce monoclonal antibodies to monomorphic or polymorphic determinants of HLA molecules were obtained from American Type Culture Collection (ATCC; Rockville, MD).[39]

Purification of $CD34^+Thy\text{-}1^+Lin^-$ cells from BM. Human adult BM (ABM) cells from normal donors were pre-enriched for $CD34^+$ cells using a magnetic bead selection device (SyStemix). $CD34^+$ cells were also selected from BM from two multiorgan donors and frozen before use. $CD34^+$ cells were incubated for 10 minutes on ice with 2 mg/mL heat-inactivated human gamma globulin (Gamimune; Miles Inc, Elkhart, IN) to block nonspecific Fc binding. Subsequently, the cells were washed with staining buffer (SB). SB contained Hank's Balanced Saline Solution (JRH Biosciences, Lenexa, KS), 0.5% bovine serum albumin (Sigma), and 10 mmol/L HEPES (Sigma). Cells were stained for 30 minutes on ice with anti-CD34-SR (6 μg/mL), anti–Thy-1-PE (10 μg/mL), and the lineage panel of FITC-conjugated antibodies. Appropriate isotype controls were used, as described above. Cells were then washed with SB and resuspended at a concentration of 10^6/mL in SB containing 1 μg/mL propidium iodide (PI; Molecular Probes Inc, Eugene, OR). A Vantage fluorescence-activated cell sorter (FACS; Becton Dickinson Immunocytometry Systems, San Jose, CA) was used to sort live (PI^{lo}) $CD34^+Thy\text{-}1^+Lin^-$ cells. The sorts were reanalyzed to assure clean separation of cell subpopulations.

PKH26 fluorescent dye labeling. Cells were washed with protein-free PBS. The PKH26 dye (Sigma) was diluted 1:250 in the kit diluent. The cell pellet was resuspended at a concentration of 10^7/mL. This cell suspension was then added to an equal volume of PKH26 and incubated for exactly 4 minutes at room temperature (RT). An equal volume of fetal bovine serum (FBS; Gemini BioProducts, Calabasas, CA) was then added and incubated for an additional 1 minute at RT. An equal volume of Iscove's modified Dulbecco's medium (IMDM) containing 10% FBS was then added. The cells were counted and then centrifuged. The pellet was resuspended at a concentration of 10^5/mL in IMDM/10% FBS with and without cytokines for short-term suspension culture. The cells were plated in round-bottom 96-well plates at 100 μL/well.

Short-term suspension culture. PKH26-labeled cells were cultured for 6 days at 10^4 cells/100 μL of medium (IMDM, 10% FBS) in round-bottom 96-well plates in suspension cultures containing different cytokine combinations. The cytokines used included IL-3 (10 ng/mL), IL-6 (10 ng/mL), LIF (50 ng/mL; Novartis, Basel, Switzerland), TPO (10 to 15 ng/mL; R&D Systems, Minneapolis, MN), KL (50 to 75 ng/mL), and FL (50 to 75 ng/mL; SyStemix). Cell numbers were determined using a hemocytometer and trypan blue to exclude dead cells.

FACS analysis of cultured cells. A fraction of PKH26-labeled cells to be used as control was kept overnight at 37°C in the absence of cytokines to remove unstably incorporated dye as well as antibodies bound to the surface and was then stained with anti-CD34-FITC. The settings (PKH26 v CD34-FITC) of the Vantage cell sorter were determined using these cells that we called control day 0 (D0). At day 6 (D6), the wells were pooled and cells were counted and stained with anti-CD34-FITC antibody after incubation with Gamimune. Cell division was measured by loss of PKH26 dye fluorescence and primitiveness by retention of the CD34 cell surface marker (Fig 1).

Undivided (PKH^{hi}) and divided (PKH^{lo}) subpopulations of $CD34^{hi}$ cells were purified from 6-day cultures containing IL-3, IL-6, and LIF or TPO and KL to determine if PHP numbers were maintained or increased within the population of $CD34^{hi}$ cells that had undergone division. Figure 2 shows typical gates used for FACS sorting from representative experiments. In each experiment, control cells were cultured without cytokines and then stained with the irrelevant mouse IgG_1-FITC to set the gates. One example of a control stain is shown for the TPO, KL, and FL combination. For this cytokine combination, all cells were PKH^{lo} and these were divided into $CD34^{hi}$ and $CD34^{lo/-}$ subsets, which were placed into the CAFC assay to determine the PHP frequency and multilineage potential of the cells postdivision.

CAFC assay. A proportion of the cells was cultured at limiting dilution in the CAFC assay as described previously[34]. Briefly, cells were seeded in 96-well plates preseeded with a murine stromal cell line (Sys-1) in 1:1 IMDM/RPMI medium (JRH BioSciences, Woodland, CA) containing 1 mmol/L sodium pyruvate (JRH BioSciences), 5×10^{-5} mol/L 2-mercaptoethanol (Sigma), and 10% FBS. Limiting dilution ranged from 100 cells per well to 0.78 cells per well. After 5 weeks, wells containing cobblestone areas were enumerated and CAFC frequency of the cell population was calculated using maximum likelihood estimation with SAS software[40]. The statistical significance of CAFC frequency difference between cultured cell populations was determined by ANOVA. Statistical significance of CAFC number difference between cultured and starting cell populations was determined using the Student's *t*-test. Representative wells containing cobblestone areas (at least 10 per sample group) were individually analyzed by FACS for the presence of $CD33^+$ immature myeloid, $CD19^+$ B-lymphoid, and $CD34^+$ progenitor cell populations to estimate the multilineage potential of the original cells.

SCID-hu bone assay. The SCID-hu bone assay was performed as previously described[34,39]. C.B-17 scid/scid mice were used as recipients of human fetal bone grafts. First, limiting dilution analysis was performed to determine the dose of $CD34^+Thy\text{-}1^+Lin^-$ cells that reliably gives donor reconstitution in the SCID-hu bone model. HLA-mismatched fetal bone grafts were injected with cell doses ranging from 1,000 to 30,000 $CD34^+Thy\text{-}1^+Lin^-$ cells per graft into mice that received whole body irradiation (400 rad) shortly before cell injection. To achieve a sufficient number of grafts at each dose, four tissue donors were used in four separate experiments. Eight weeks after injection, the bone grafts were recovered and the BM cells harvested and analyzed for donor cell engraftment using FITC conjugates of allotype-specific HLA antibodies versus PE-conjugated anti-CD19, anti-CD33, and anti-CD34. Total human cells were detected with W6/32-PE (antihuman HLA class I major histocompatibility complex [MHC] molecule monomorphic determinant). Cells were analyzed on a FACScan analyzer (Becton Dickinson Immunocytometry Systems). Grafts having at least 1% of hematopoietic cells bearing donor HLA antigen were considered positive. The percentage of grafts showing donor reconstitution was assayed for each cell dose tested. At five times the limit dose, or 10,000 cells, donor reconstitution was observed in all grafts.

Uncultured BM $CD34^+Thy\text{-}1^+Lin^-$ as well as $CD34^{hi}$ PKH^{lo} and $CD34^{lo/-}$ PKH^{lo} cells from D6 cultures in TPO, KL, and FL were sorted and injected (10,000 cells per graft) into SCID-hu bone grafts. Eight weeks after injection, the bone grafts were analyzed for engraftment of donor $CD33^+$, $CD19^+$, and $CD34^+$ cells.

Kinetics of cell division. A fraction of PKH26-labeled $CD34^+Thy\text{-}1^+Lin^-$ cells was kept overnight at 37°C in the absence of cytokines and then stained with anti-CD34-FITC. The settings (PKH26 v CD34-FITC) of the FACS Calibur (Becton Dickinson Immunocytometry Systems) were determined using these cells (control D0). Short-term suspension cultures of PKH26-labeled $CD34^+Thy\text{-}1^+Lin^-$ cells were set up in different cytokine combinations, as described above. Cells were stained on D2, D4, and D6 with anti-CD34 (HPCA-2)-FITC and anti-Thy-1-

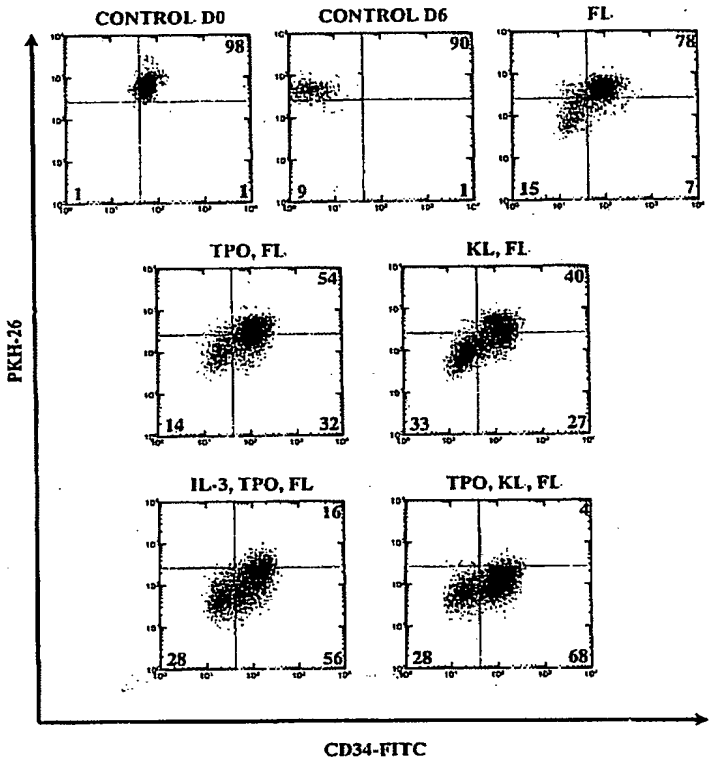


Fig 1. FACS analysis of CD34⁺Thy-1⁺Lin⁻ cells cultured for 6 days in various cytokine combinations containing FL. Numbers are percentages of cells in each quadrant from a representative experiment. Loss of PKH26 fluorescence indicates cell division. Loss of CD34 expression indicates differentiation. Control cells were cultured overnight (D0) or for 6 days (D6) without cytokines and quadrants were set using live-gated undivided cells. The percentages of undivided cells (UR and UL quadrants) are combined.

Cy5 (GM201-Cy5 conjugated at SyStemix) and analyzed on the FACS Calibur

RESULTS

Increase of total cell number and of CD34⁺ cell number. In the present study, we examined the effects of single, double, and triple cytokine combinations in 6-day suspension cultures of PKH26-labeled BM CD34⁺Thy-1⁺Lin⁻ cells. Previous studies using PKH26 did not show any detrimental effects of PKH26 labeling on cellular function.[36,38] As shown in Table 1, single cytokines did not increase the number of CD34⁺ or total cells. Combinations of two cytokines of TPO, KL, and FL maintained the CD34⁺ cell number with a slight increase (1.7-fold) in total cell number. Among those tested, the combination of three cytokines, TPO, KL, and FL, induced the highest increases of both total cell (4.7-fold) and of CD34⁺ cell number (3.4-fold). The three-factor combination IL-3, IL-6, and LIF did not stimulate an increase in total cell number.

Comparison of different cytokine combinations containing FL. We examined the effect of FL alone and in combination with one or two other cytokines in three to six experiments. In Fig 1, we demonstrate how the quadrants were set on the control unstimulated cells and show dot plots from a representative experiment. When cultured in FL alone, most CD34⁺Thy-1⁺Lin⁻ cells remained undivided (78%). Sixty-eight percent (mean 73%) of postdivision cells lost CD34 expression. The addition of KL to FL reduced by half the percentage of undivided cells (to 40%), and 55% (mean 58%) of postdivision cells lost CD34 expression. The addition of TPO to KL and FL stimulated much greater division (4% remained undivided) with loss of CD34 on only 29% (mean 27%) of postdivision cells. With other combinations containing TPO, eg, TPO and FL or TPO, IL-3, and FL, we also observed that loss of CD34 expression only occurred on about 30% of postdivision cells. We had previously shown that IL-3 induces not only division, but also differentiation (CD34 loss) of human HSC.[11] The addition of TPO seems not only to contribute to greater cell division but also to overcome the effect of IL-3 to promote differentiation.

To determine whether retention of CD34^{hi} expression postdivision correlated with retention of functional PHP, CD34/PKH26 subsets were purified postculture in three different cytokine conditions and assayed for CAFC frequency. CD34^{hi} PKH26^{lo} and CD34$^{lo/-}$ PKH26^{lo} subsets from TPO, KL, and FL cultures were also assayed for in vivo SCID-hu bone repopulating activity.

FACS sorting of cultured cell subpopulations subdivided by PKH26 fluorescence and CD34 staining. In subdividing CD34^{hi} cells based on cell division, we tried to exclude the CD34$^{lo/-}$ subpopulation, because it is known that CAFC are contained mainly within the CD34^{hi} population,[37] as confirmed in Table 2. The majority of cells in IL-3, IL-6, and LIF did not divide (mean 75%) by day 6; therefore, we sorted CD34^{hi} PKH^{hi} versus CD34^{hi} PKH^{lo} (mean 7.5%; Fig 2). The same cell

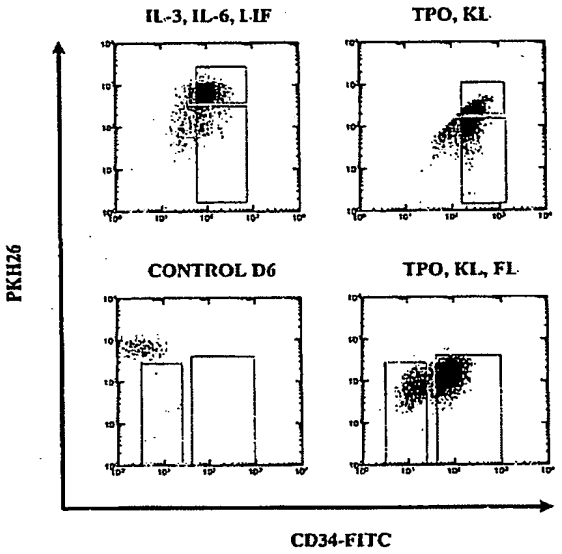


Fig 2. FACS sort gates based on PKH26 versus CD34 fluorescence. After 6 days of culture of CD34⁺Thy-1⁺Lin⁻ cells in different combinations of cytokines, cells were stained with anti-CD34-FITC. Sort gates shown are on live (PI low) cells. These were set based on the PKH26 profile of live unstimulated control cells. Each cytokine condition required a different tissue for sorting and therefore sort gates varied accordingly.

populations were sorted from cultures with TPO and KL in which a mean of 53% of cells remained undivided and a mean of 23% of cells were $CD34^{hi}$ PKH^{lo}. In TPO, KL, and FL, all the cells had divided; therefore, we sorted for $CD34^{hi}$ PKH^{lo} (mean 71%) versus the $CD34^{lo/-}$ PKH^{lo} (mean 26%) population of differentiated postdivision cells.

Analysis of CAFC frequencies and phenotype of cobblestone areas. The PHP activity of the sorted CD34/PKH26 subpopulations of cultured cells was estimated in vitro by use of the CAFC assay, comparing the CAFC frequencies with the starting population of $CD34^{+}Thy\text{-}1^{+}Lin^{-}$ cells. The mean frequencies of CAFC within the starting population of $CD34^{+}Thy\text{-}1^{+}Lin^{-}$ cells ranged from 1/21 to 1/46 (95% confidence limits, 1/16 to 1/52; Table 2). Because of the limited number of cells obtained from each fresh BM, only one cytokine combination could be tested per experiment, giving rise to some tissue variation. In the case of IL-3, IL-6, and LIF, the undivided $CD34^{hi}$ PKH^{hi} subpopulation remained primitive, retaining the same mean CAFC frequency as the preculture $CD34^{+}Thy\text{-}1^{+}Lin^{-}$ population. However, the frequency of CAFC within the small $CD34^{hi}$ PKH^{lo} subpopulation had decreased 21-fold to a mean of 1/440 (1/249 to 1/813).

In addition, we evaluated the ability of cultured cell subpopulations to give rise to both myeloid and B-lymphoid cells in long-term stromal culture. Detection of $CD34^{+}$ cells in 5-week cobblestone areas suggests retention of primitiveness among the cell subpopulations assayed. Analysis of a minimum of 10 small cobblestone areas generated from this population showed that divided $CD34^{hi}$ cells from cultures containing IL-3, IL-6, and LIF gave rise to only $CD33^{+}$ myeloid cells (Table 3).

Table 1. Comparison of Fold Increase of Total Cells and $CD34^{+}$ Cells in 6-Day Cultures in Single Cytokines or in Cytokine Combinations

Cytokines	Fold Increase of Total Cells	Fold Increase of $CD34^{+}$ Cells
TPO	0.30	0.28
FL	0.72 ± 0.10	0.55 ± 0.06
KL	0.67 ± 0.15	0.56 ± 0.09
TPO, KL*	1.69 ± 0.47	1.02 ± 0.12
TPO, FL	1.67 ± 0.60	1.41 ± 0.50
KL, FL	1.67 ± 0.14	1.08 ± 0.09
TPO, KL, FL*	4.71 ± 1.50	3.43 ± 1.07
IL-3, TPO, FL	3.17 ± 1.10	2.28 ± 0.75
IL-3, IL-6, LIF*	0.86 ± 0.10	0.68 ± 0.08

*Divided and undivided CD34 subpopulations from these cultures were analyzed in the CAFC assay. The values for TPO, KL, FL represent the means ± SEM of six experiments. All other conditions (except TPO [1] and KL [2]) are means ± SEM of three experiments.

Table 2. Mean CAFC Frequencies of CD34/PKH26 Cell Subsets From 6-Day Cultures

Cytokines	Cell Population	Day of Culture	CAFC Frequency
IL-3, IL-6, LIF	$CD34^{-}Thy\text{-}1^{-}$	0	1/21 (1/16-1/26)
	$CD34^{hi}PKH^{hi}$	6	1/21 (1/15-1/23)
	$CD34^{hi}PKH^{lo}$	6	1/440 (1/249-1/813)
TPO, KL	$CD34^{-}Thy\text{-}1^{-}$	0	1/33 (1/28-1/46)
	$CD34^{hi}PKH^{hi}$	6	1/44 (1/37-1/56)
	$CD34^{hi}PKH^{lo}$	6	1/75 (1/59-1/89)
TPO, KL, FL	$CD34^{-}Thy\text{-}1^{-}$	0	1/46 (1/41-1/52)
	$CD34^{hi}PKH^{hi}$ *	6	
	$CD34^{hi}PKH^{lo}$	6	1/42 (1/33-1/59)
	$CD34^{lo/-}PKH^{lo}$	6	1/2,898 (1/1,743-1/13,415)

CAFC frequencies at 5 weeks were calculated using maximum likelihood estimation with SAS software [40]. The 95% confidence limits are shown in parentheses. Values are the mean of six experiments for TPO, KL, FL and the mean of two experiments for the other cytokine combinations.

*Not determined because all cells had undergone division after 6 days of culture in TPO, KL, FL.

Table 3. The Ability to Give Rise to B-Lymphoid and $CD34^{+}$ Progenitor Cells in Long-Term Stromal Cultures Was Preserved for $CD34^{hi}$ Cells That Had Divided in TPO, KL, and FL

			Percentage of Positive Wells	
Cytokines	Cell Population	Day of Culture	$CD19^{+}$ B-lymphoid	$CD34^{+}$ Progenitors
IL-3, IL-6, LIF	$CD34^{-}Thy\text{-}1^{-}$	0	71.8 ± 8.2	63.2 ± 26.8
	$CD34^{hi}PKH^{hi}$	6	35.9 ± 2.6	40.4 ± 9.6
	$CD34^{hi}PKH^{lo}$	6	0	0
TPO, KL	$CD34^{-}Thy\text{-}1^{-}$	0	58.2 ± 19.7	79.1 ± 9.9
	$CD34^{hi}PKH^{hi}$	6	71.8 ± 5.1	28.2 ± 5.1
	$CD34^{hi}PKH^{lo}$	6	63.4 ± 0.9	3.6 ± 3.6
TPO, KL, FL	$CD34^{-}Thy\text{-}1^{-}$	0	41.5 ± 16.5	66.9 ± 16.8
	$CD34^{hi}PKH^{lo}$	6	54.2 ± 8.5	49.0 ± 27.1
	$CD34^{lo/-}PKH^{lo}$	6	ND	ND

Wells were scored positive if greater than 1% of cells were positive for the surface marker. All cobblestone areas analyzed contained $CD33^{+}$ myeloid cells. Values are the means ± SEM for two experiments (IL-3, IL-6, LIF and TPO, KL) or six experiments (TPO, KL, FL).

Abbreviation: ND, not determined due to no or a limited number of wells containing cobblestone areas.

After culture with TPO and KL, the undivided CD34^{hi} PKH^{hi} cells again had a similar CAFC frequency to the uncultured CD34$^+$Thy-1$^+$Lin$^-$ population. In these conditions, the mean frequency of CAFC in the divided CD34^{hi} PKH^{lo} subpopulation was reduced 2.3-fold, compared with the starting cell population (Table 2). CD34^{hi} PKH^{lo} cells retained the potential, at limiting dilution, to give rise to CD19$^+$ B-lymphoid, CD33$^+$ myeloid, and CD34$^+$ progenitor cells after 5 weeks of culture in the CAFC assay. However, the proportion of wells containing greater than 1% CD34$^+$ cells was reduced 22-fold compared with the starting cell population (Table 3). For each cell population, all cobblestone areas analyzed contained CD33$^+$ myeloid cells.

The mean values for six experiments with the combination of TPO, KL, and FL are shown in Table 2. The mean CAFC frequency remained the same in the CD34^{hi} PKH^{lo} subpopulation, compared with the starting CD34$^+$Thy-1$^+$Lin$^-$ cell population. These cells also, at limiting dilution, retained their ability to give rise to CD19$^+$ B-lymphoid progenitors, CD33$^+$ myeloid cells, and CD34$^+$ progenitor cells. CD19$^+$ cells were observed in a similar proportion (~50%) of cobblestone areas examined for both the uncultured CD34$^+$Thy-1$^+$Lin$^-$ cells and postculture CD34^{hi} PKH^{lo} cells. Forty-nine percent of cobblestone areas generated from CD34^{hi} PKH^{lo} cells contained CD34$^+$ cells, as compared with 67% for the uncultured CD34$^+$Thy-1$^+$Lin$^-$ cells (Table 3). As expected, the CD34$^{lo/-}$ PKH^{lo} cells had very low CAFC frequency (mean 1/3,000).

Increase in CAFC numbers among total and CD34^{hi} PKH^{lo} cells. We compared the increase of CAFC numbers from CD34$^+$Thy-1$^+$Lin$^-$ cells in different culture conditions (Fig 3).

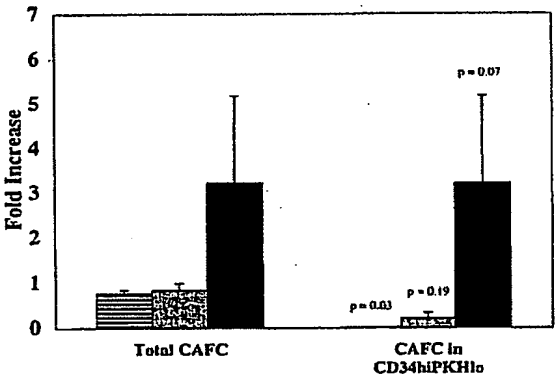


Fig 3. Increase of postdivision CAFC numbers during 6 days of culture in TPO, KL, and FL. Increased numbers of CAFC (CD34^{hi} PKH^{hi} and PKH^{lo}) were determined by dividing the number at day 6 by the number placed in culture at day 0 (left-hand columns). On the right, columns show the fold increase in numbers of CAFC within the CD34^{hi} PKH^{lo} (postdivision) population, compared with the number within the CD34$^+$Thy-1$^+$Lin$^-$ population placed in culture at D0. There was a mean 3.2-fold increase (range 1- to 7.6-fold) in postdivision CAFC in TPO, KL, and FL cultures. Data shown for IL-3, IL-6, and LIF as well as TPO and KL are the means of two experiments. Data for TPO, KL, and FL are the means of six experiments (4 normal and 2 multi-organ donor BM). Error bars show the SEM and *P* values indicate the significance of the change in CAFC number from D0 to D6 (▤) IL-3, IL-6, and LIF; (□) TPO and KL; (■) TPO, KL, and FL.

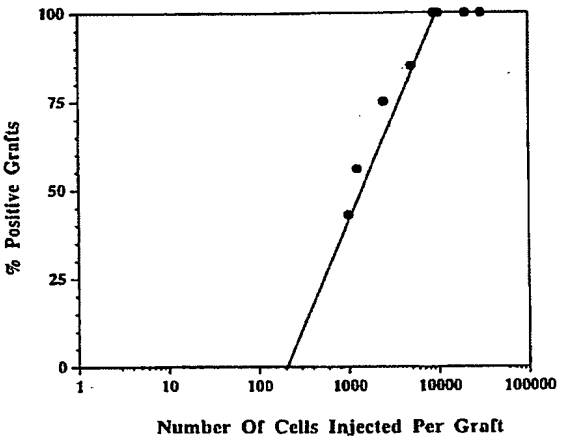


Fig 4. Titration of BM CD34$^+$Thy-1$^+$Lin$^-$ cells in the SCID-hu bone model. Bone grafts were injected with a range of doses of CD34$^+$Thy-1$^+$ Lin$^-$ cells (1,000 to 30,000) per graft. Data are the mean of four separate experiments from 4 different BM donors. Donor reconstitution means that greater than 1% of hematopoietic cells were positive for donor HLA antigen.

Of the three different cytokine combinations analyzed, only TPO, KL, and FL increased the mean number of total cells, CD34$^+$ cells, and CAFC (Table 1 and Fig 3). If we compare the number of CAFC within the CD34^{hi} PKH^{lo} population with the original number of CAFC placed in culture, we can see that only in TPO, KL, and FL were CAFC numbers increased among cells that had divided (mean 3.2-fold), although values ranged from maintenance to a 7.6-fold increase. The CAFC number among divided CD34^{hi} cells at day 6 was not significantly different from the number measured among CD34$^+$Thy-1$^+$Lin$^-$ cells at day 0 for TPO and KL cultures ($n = 2$, $P = .19$), but increased CAFC number among CD34^{hi} PKH^{lo} cells in TPO, KL, and FL cultures approached statistical significance ($n = 6$, $P = .07$). The number of measurable CAFC among divided CD34^{hi} cells from IL-3, IL-6, and LIF cultures had significantly decreased ($n = 2$, $P = .03$). All CAFC detectable in D6 IL-3, IL-6, and LIF cultures were derived from undivided CD34^{hi} cells.

Dose of uncultured CD34$^+$Thy-1$^+$Lin$^-$ cells that gives reconstitution of 100% of SCID-hu bone grafts.[34,39] The percentage of grafts showing donor reconstitution at each CD34$^+$Thy-1$^+$Lin$^-$ cell dose tested is shown in Fig 4. Using Poisson distribution analysis, the frequency of SCID-hu bone repopulating cells was 1 per 2,000 CD34$^+$Thy-1$^+$Lin$^-$ cells. At five times this limit dose or 10,000 cells, donor reconstitution was observed in 100% of grafts.

Engraftment of CD34^{hi} PKH^{lo} cells from 6-day culture in TPO, KL, and FL in SCID-hu bone. CD34^{hi} PKH^{lo} cells from 6-day cultures of CD34$^+$Thy-1$^+$ Lin$^-$ cells in TPO, KL, and FL clearly contained increased numbers of CAFC. In addition, we asked whether the same cell population retained its ability to repopulate human bone in vivo, using the SCID-hu bone assay[34,39]. To obtain sufficient cells, we purified CD34$^+$Thy-1$^+$Lin$^-$ cells from cryopreserved BM CD34$^+$ cells isolated

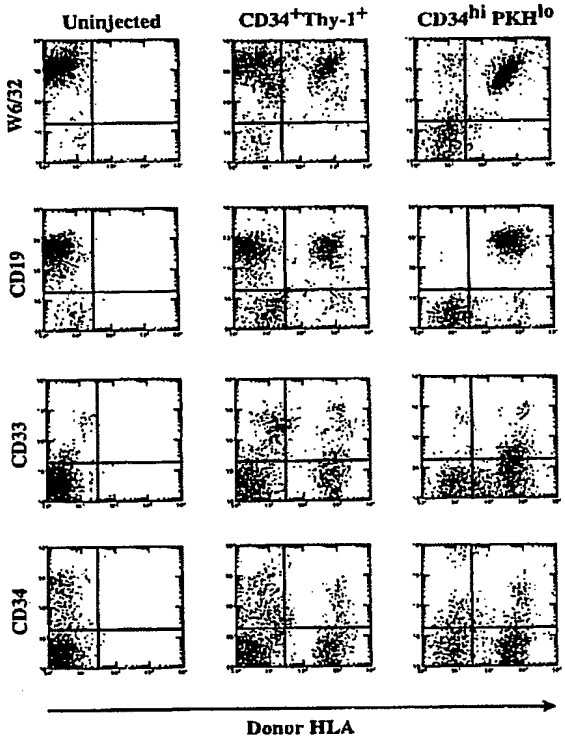


Fig 5. CD34+ cells that have divided during 6 days of culture in TPO, KL, and FL retain their capacity for in vivo marrow repopulation in the SCID-hu bone assay. Uncultured CD34+Thy-1+Lin- BM cells and CD34hi PKHlo and CD34lo/- PKHlo cells from D6 TPO, KL, and FL cultures were injected into SCID-hu bone grafts (10,000 cells per graft). FACS analysis at 8 weeks showed multilineage marrow repopulation by both the uncultured cells and the CD34hi cells postdivision in culture (PKHlo). The x-axis shows staining for donor HLA allotype. The y-axis shows staining for total human cells (W6/32, antihuman class I MHC) or lineage markers.

from multiorgan donors. Uncultured CD34+Thy-1+Lin- cells and CD34hi PKHlo as well as CD34lo/- PKHlo cells from D6 TPO, KL, and FL cultures were injected into the fetal human bone grafts. Ten thousand cells were injected per graft, because this cell dose provides consistent engraftment of uncultured BM CD34+Thy-1+Lin- cells (Fig 4).

Cultured CD34hi PKHlo cells engrafted to a similar level as the uncultured population of CD34+Thy-1+Lin- cells (4 of 4 grafts; Fig 5 and Table 4). In experiment A (Table 4), the mean percentage of donor cells was 34.3% ± 22.3% for CD34hi PKHlo cells, comparable with 25.0% ± 13.5% for uncultured CD34+Thy-1+Lin- cells. FACScan analysis shows that multilineage engraftment occurred in both cases, because the cells isolated from the bones after 8 weeks included donor B-lymphoid (CD19+), myeloid (CD33+), and progenitor cells (CD34+; Fig 5). Cells of the CD34lo/- PKHlo subpopulation engrafted in 1 of 4 bones and did not give rise to cobblestone areas in vitro. This single engraftment (3.5% ± 5.3% donor) could have been due to a low level of contamination (6%, seen in reanalysis) of the CD34lo/- population with CD34hi cells.

In a second experiment (B), in which the contamination of CD34lo/- with CD34hi cells was less than 2%, we could show that 0 of 4 bones injected with the CD34lo/- PKHlo subset engrafted, but 4 of 4 grafts injected with CD34hi PKHlo cells from D6 TPO, KL, and FL cultures again showed multilineage engraftment, with a mean of 59.0% ± 12.0% donor cells (Table 4). This confirms that, after 6 days of culture, the in vivo marrow repopulating capacity of CD34+Thy-1+Lin- cells is retained within the CD34hi population postdivision in TPO, KL, and FL.

Comparison of the kinetics of cell division in TPO, KL, and FL and IL-3, IL-6, and LIF. For retroviral gene transduction of PHP and HSC, it will be important to know the timepoint where division is maximal, but differentiation is minimal. We, therefore, examined CD34 retention (primitiveness) and PKH26 loss (division) by CD34+Thy-1+Lin- cells at D2, D4, and D6. In addition, we stained the cells to follow Thy-1 expression as a marker of PHP (blue in Fig 6A). We chose to compare the cytokine combination of IL-3, IL-6, and LIF with TPO, KL, and FL, which in our study stimulated greater division of PHP with retention of primitive phenotype. A representative experiment (of 3 experiments) is shown in Fig 6.

In IL-3, IL-6, and LIF, 65% (mean 80%) of cells remained undivided at D6. Forty-three percent of postdivision cells lost expression of CD34 and also appeared to lose Thy-1 expression (Fig 6A). Only 12% (mean 7%) of cells had divided by D4. In TPO, KL, and FL, most cells underwent the first cell division between D2 and D4, because only 5% (mean 7%) of cells lost

Table 4. CD34+ Cells That Have Divided During 6 Days of Culture in TPO, KL, and FL Retain Their Capacity for Marrow Repopulation In Vivo in the SCID-hu Bone Assay

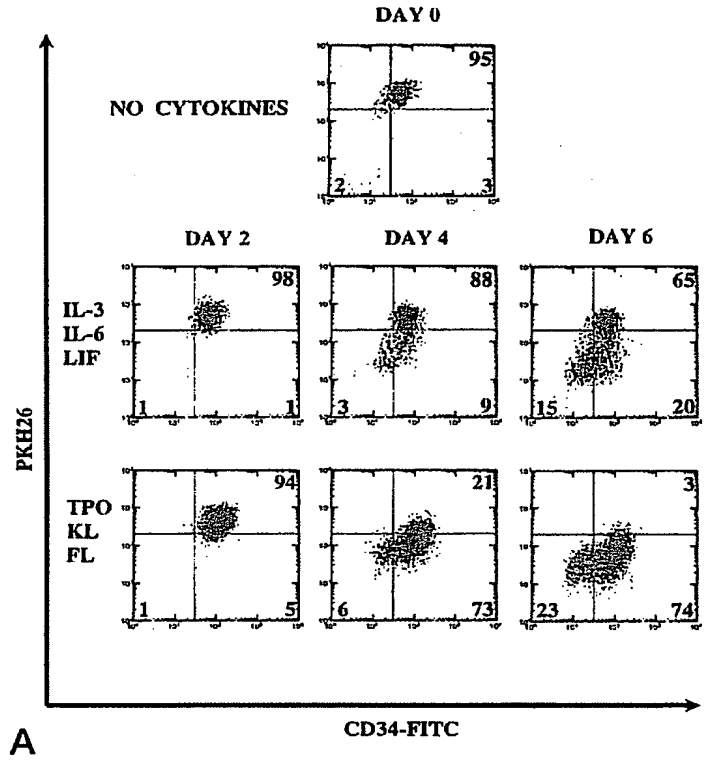
Cell Population	Experiment	CAFC Frequency	Positive Grafts	% Donor	% CD19+	% CD33+	% CD34+
CD34+Thy-1-	A	1/106	4/4	25.0 ± 13.5	22.0 ± 12.5	4.8 ± 2.8	4.5 ± 0.5
	B	1/38	ND*	ND	ND	ND	ND
CD34hiPKHlo	A	1/36	4/4	34.3 ± 22.3	31.3 ± 19.8	6.7 ± 6.2	3.9 ± 3.4
	B	1/26	4/4	59.0 ± 12.0	58.0 ± 11.5	1.9 ± 0.6	5.0 ± 1.0
CD34lo/-PKHlo	A	<1/6,600†	1/4	3.5 ± 5.3	3.3 ± 4.9	1.3 ± 1.8	ND
	B	1/932	0/4	0	0	0	ND

Ten thousand cells were injected per bone graft. Errors shown are SEM. Grafts were analyzed 8 weeks after injection for donor cells expressing the HLA marker of the injected cells.

Abbreviation: ND, not determined.

*Mice used for injection of uncultured CD34-Thy-1-Lin- cells in experiment B died before analysis of the grafts.

†No CAFC detected among 6,600 cells plated.



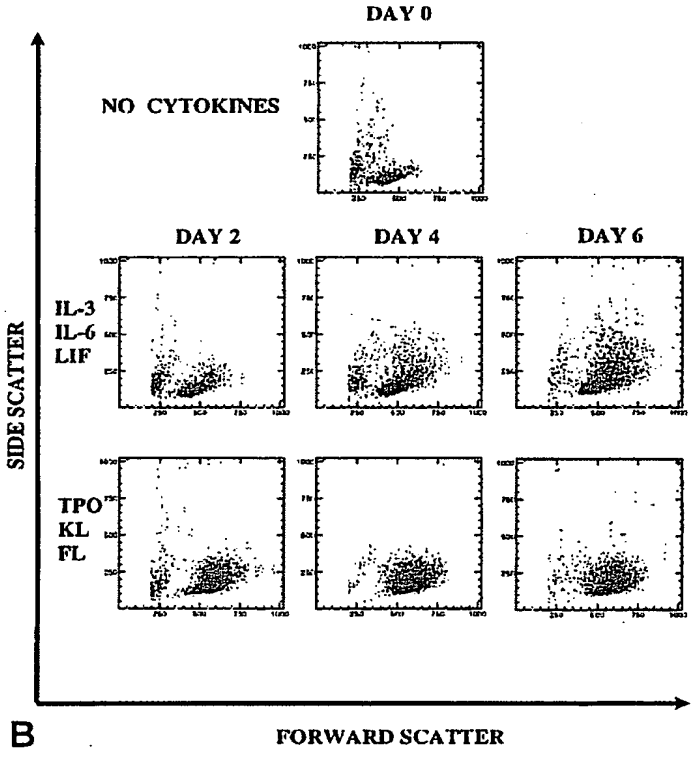


Fig 6. Kinetics of CD34⁺Thy-1⁺Lin⁻ cell division and differentiation during 6 days of culture. Comparison between IL-3, IL-6, and LIF and TPO, KL, and FL. (A) PKH26 versus CD34 fluorescence. Live Thy-1⁺ cells are shown in blue and live Thy-1⁻ cells are shown in red. Percentages of PKH^{hi} and $CD34^{hi}$ PKH^{lo} and $CD34^{lo}$ $PKH^{lo/-}$ cell subsets are shown for this representative experiment (of 3 experiments). (B) forward versus side scatter. Live Thy-1⁺ cells are shown in blue, and live Thy-1⁻ cells are shown in red.

PKH26 fluorescence by D2, yet already by D4, 73% (mean 75%) of the cells lost PKH26 fluorescence CD34^{hi} expression was retained on 92% of postdivision cells By D6, 97% of the cells had divided with retention of CD34^{hi} expression on 76% of these cells Clearly, Thy-1 expression (blue) was retained on the CD34^{hi} PKH^{lo} cells from TPO, KL, and FL D6 cultures, which is consistent with the retention of PHP and in vivo engraftment activity demonstrated within this cell population In Fig 6B, the early myelopoietic effects of IL-3 could be detected as an increase in side scatter of the cultured cells In contrast, in TPO, KL, and FL there was less cell death and the scatter profiles indicate a predominance of blast morphology during the 6 days of suspension culture, consistent with less differentiation The increase in size (FSC) of the Thy-1$^+$ cell subset in TPO, KL, and FL is shown in blue (Fig 6B)

DISCUSSION

TPO synergizes with KL to promote multilineage proliferation of both human[10,11,29,30] and mouse HSC[26-28,35] FL is a ligand for the flk2/flt3 tyrosine kinase receptor[18,19] that seems to have a unique expanding effect on human peripheral blood long-term culture-initiating cells (LTC-IC)[24] TPO has been shown to synergize with both KL and FL to enhance both the number and size of clones formed by murine Sca1$^+$ Lin$^-$ progenitor cells[35] FL appears to partially replace the requirement for stroma to maintain the human long-term repopulating HSC during gene transduction,[13] whereas IL-3, IL-6, and KL were insufficient[4]

We have previously described the ability of TPO[25] to increase the number of human CD34$^+$ cells detectable after long-term culture[11] We also showed that TPO and KL could synergize to drive division of primitive human BM CD34$^+$Lin$^-$Rhodamine123^{lo} cells with retention of CD34 expression[11] The question remained whether the CD34$^+$ cells that had undergone division (PKH^{lo}) retained primitive functional characteristics In the present study, culture with TPO, KL, and FL stimulated virtually all CD34$^+$Thy-1$^+$Lin$^-$ cells to divide by day 6, with a 3 4-fold increase in numbers of CD34$^+$ cells

Expansion of LTC-IC (mean, 7 5-fold) has previously been described from whole BM mononuclear cells using 14-day continuous perfusion culture bioreactors containing a stromal layer[41] In addition, Petzer et al[42] have shown 30-fold expansion of LTC-IC within 10 days, starting with a highly purified HSC population and using a combination of 6 cytokines, including KL and FL In our study, we have used static cultures containing only 3 cytokines (TPO, KL, and FL) and observed a mean 3 2-fold increase of CAFC numbers within 6 days within a population that has been shown to be postdivision, based on loss of PKH26 fluorescence The minor population of CD34^{hi} PKH^{lo} postdivision cells from cultures with IL-3, IL-6, and LIF were found to contain very few CAFC, in contrast to undivided CD34^{hi} PKH^{hi} cells, which retained CAFC at high frequency[38] Only by using PKH26 to separate undivided and divided CD34^{hi} cells could we show that CAFC in cultures containing IL-3, IL-6, and LIF represent undivided cells, whereas CAFC from cultures with TPO, KL, and FL had all been generated de novo by cell division In the study by Petzer et al,[42] only TPO and FL when used alone stimulated a net increase of LTC-IC from CD34$^+$CD38$^-$ cells within 10 days TPO and FL have also been shown to induce extensive renewal with little differentiation of cord blood LTC-IC ex vivo[31] In our system, in TPO and KL or in TPO, KL, and FL, CD34^{hi} PKH^{lo} cells showed retention of the ability to give rise to B-lymphoid as well as early myeloid cells in 5-week stromal cultures

Long-term bone repopulating cells are likely to be more primitive than the majority of those that read out in the 5-week CAFC assay Our demonstration that CD34^{hi} PKH^{lo} cells from 6-day cultures with TPO, KL, and FL retained the ability to give a high level of engraftment (both B-lymphoid and myeloid) at 8 weeks in our SCID-hu bone transplant model indicates that the multipotency and engraftment potential of CD34$^+$Thy-1$^+$Lin$^-$ cells was preserved during cell division in vitro Ex vivo expansion of HSC that retains the ability to engraft may allow reduction of periods of cytopenia when numbers of such cells are limiting for autologous transplantation, as well as production of sufficient numbers to overcome allogeneic transplant barriers

Levels of gene transfer into pluripotent HSC remain low, potentially due to the failure to induce division of the majority of primitive HSC within the short transduction period TPO has been proposed to shorten the G_0 period of dormant murine progenitor cells[27] We suggest that the rapid division of PHP stimulated by TPO, KL, and FL may be due to this combination of factors driving quiescent PHP to exit G_0, as well as shortening the G_1 phase of the cell cycle[43,44] Based on these premises, an optimal time to achieve integration of retroviral vectors into dividing HSC would, therefore, be between day 2 and day 4, using TPO, KL, and FL Further studies will be necessary to determine whether achieving maximal division of HSC will be sufficient to overcome the barrier to transducing pluripotent long-term engrafting stem cells

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